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Acanthamoeba and the bacterial pathogen interactions

By

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Declaration

I declare that this thesis has been composed by myself, and that the work is my own unless otherwise stated; significant contributions from research groups I have been a part of are clearly indicated. This work has not been submitted for any other degree or professional qualification.

Signed…….

Abstract

The present study investigates *Acanthamoeba*-bacteria interaction and how this relation can influence human health aiming at the influence of bacteria on *Acanthamoeba* in terms of their isolation and diversity, and the effect of *Acanthamoeba* on bacteria focusing on two emerging human bacterial pathogens *Arcobacter butzleri* and *Rhodococcus equi*. To first objective was investigated by the test question "can the presence of a particular type of bacteria play role in the diversity of *Acanthamoeba* by masking and/or favouring certain genotypes of *Acanthamoeba*?"

To answer this, two different bacteria the Gram+ve *Enterococcus* the Gram-ve *Arcobacter* were used as food source for isolation of *Acanthamoeba* from 102 soil samples while *E. coli* was used as control. It was found that the presence of different bacteria could affect the isolation of genotypes specially the subgroups and subtypes of *Acanthamoeba* as manifested by greater diversity of 18S rRNA sequences of *Acanthamoeba* isolated from environmental samples on *Arcobacter* (Arc) and *Enterococcus* (Ent) than those isolated on *E. coli* (Eco). The Eco isolates consisted of only T4>T11=T13 compared to Ent isolates with T4>T16>T13/16 and the Arc isolates which comprised of T4>T2>T2/6=T13>T13/16. The T13/16 were the intermediate sequence types with no match to any T types. There were also considerable differences among the T4 subgroups; the Eco isolates consisted of T4-A>T4-B>T4-N>T4-E>T4-D>T4-C while Ent isolates comprised of T4-A>T4-C=T4-D=T4-E=T4-N>T4-B and the Arc isolates had only T4-E>T4-A>T4-B>T4-N. In both Eco and Ent isolates 11 subtypes were recovered with T4-36 being the most abundant, however, in Arc isolates eight subtypes were recovered with T4-12 as the most abundant. The non-Eco isolates were also different in their bacterial endosymiotic profile from Eco isolates with Arc isolates having the greatest proportion of bacterial endosymbionts (15.7%) as compared to 7.8% of Eco and 12.9% of Ent isolates. Together these results indicate a prominent role of prey bacteria on favouring certain genotypes and thus compelling consideration for use of different types of bacteria for isolation of *Acanthamoeba* to help surface the masked populations as well for more realistic prevalence that will help in better designing of prevention and control strategies.

The influence of *Acanthamoeba* on bacteria was investigated for *A. butzleri* and *R. equi* both of which appeared to exploit the former as an environmental reservoir and for modulation of their pathogenic potential. *A. butzleri* which are closely related to *Campylobacter*, appeared to have a smooth interaction with *Acanthamoeba.* They were shown to be easily located through chemotaxis, readily attached and internalized using monosaccharide receptors and a complex phagocytic process, and could survive/proliferate in *Acanthamoeba* by defying the intra-vacuolar killing processes. Intracellular survival in *Acanthamoeba* did play a role in promoting the pathogenicity of these bacteria enabling them to survive more than three times longer. Co-culturing of the two organisms also seemed to benefit the bacteria but not *Acanthamoeba*. *A. butzleri* were found to be able to sense the environmental changes and thus modulate their virulence, a feature that together with selection pressure for intracellular survival in *Acanthamoeba* can cause rapid adaptation to intra-amoebal environment and enhance the pathogenic potential of these bacteria for humans and animals.

Exploitation of *Acanthamoeba* for survival was also found to be exhibited by the *Mycobacterium*-resembling Gram+ve *R. equi* by utilizing similar strategies for survival/proliferation as used for macrophages, which involved the definite presence of virulence plasmid and its activation at higher temperatures. Moreover, similar genes (*vapA*, *vapC* and *vapF*) were found to play role in intracellular survival in both the macrophages and amoeba cells. The intra-amoebal survival/proliferation capabilities of *A. butzleri* and *R. equi* appear to support the notion that free living protists like *Acanthamoeba* act as environmental

reservoirs/virulence trait selectors and are strong candidates for the “missing link” between the ecology and pathology of these emerging pathogenic pathogens.

Overall, the observations made in this study explore the vital role of *Acanthamoeba*-bacteria interaction not only mutually on each other but as a consequence the impact on human health either as a result of masked genotypes in clinical diagnosis of *Acanthamoeba* or due to environmental reservoir role of *Acanthamoeba* in selecting virulence traits of bacteria, can pose serious challenges leaving ample opportunities for more emerging bacterial pathogens. These observations call for revising the protocols for *Acanthamoeba* prevalence, eradication and control strategies.

Lay Summary

Acanthamoeba are free-living single-celled organisms that are not only found widely distributed in the environment but they are also responsible for causing serious damage to the eye and brain in humans. There are a number of different types and subtypes of *Acanthamoeba*. In the environment *Acanthamoeba* eat bacteria as food. Formerly, it was thought that the relation between *Acanthamoeba* and bacteria is merely of predator-prey in nature. However, in early 80's it was found that the bacteria like *Legionella*, which are responsible for Legionnaires' disease in humans, can not only resist killing but also they can grow inside *Acanthamoeba* with their eventual killing. Ever since then the interaction between *Acanthamoeba* and other bacteria has received growing attention. In the current study, I investigated that when *Acanthamoeba* and bacteria come in contact, how their interaction can impact mutually both these organisms.

Firstly, to study how the presence of various types of bacteria as food source can affect the *Acanthamoeba* population in terms of their types that survive better than the others, I used three different kind of bacteria (*Escherichia coli, Enterococcus* and *Arcobacter*) to separately feed *Acanthamoeba* for isolation from the soil samples collected from various parts of England and Scotland. Different *Acanthamoeba* types and subtypes were recovered for the three kind of bacteria used. This indicates that *Acanthamoeba* have preferential feeding upon bacteria and, therefore, the bacteria prevailing as food source can in turn limit the population of various types of *Acanthamoeba* in an area. These findings necessitate revising the traditional method for isolation of *Acanthamoeba*, which relies on using just one type of bacteria (*E. coli*), to consider a combination of different kinds of bacteria for isolation from environmental and clinical samples for more reliable prevalence and diagnosis of *Acanthamoeba*.

In the other half of the research, I studied how *Acanthamoeba* can affect bacteria as a result of interaction with them. I used two emerging disease-causing bacteria in humans to investigate this, that is, *Arcobacter butzleri* (which are found in the environment and also cause infection of digestive system in humans) and *Rhodococcus equi* (which live in soil but also cause lung infection primarily in foals with potential to cause disease in humans as well). The results indicated that both *A. butzleri* and *R. equi* have the potential to resist killing by *Acanthamoeba* to a certain extent. The survival of *A. butzleri* in *Acanthamoeba* enhanced their ability to resist killing by the latter. While *R. equi* were found to use similar strategies to survive in *Acanthamoeba* as well as in defence cells (macrophages). Because of the striking similarity of *Acanthamoeba* with macrophages it is possible that *Acanthamoeba* acted as training grounds for bacteria in improving their skills to resist killing inside the cells, which is required for survival in macrophages and thus causing diseases. This also explains the emergence of previously harmless environmental bacteria as the disease-causing agents in humans now.

Overall, as a consequence of their interaction, both *Acanthamoeba* and bacteria appear to be impacted by each other. Investigations of such interactions will not only help in understanding the diversity of this relation but also it will help in better designing and implementing control strategies for both the bacterial and *Acanthamoeba* infections.

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List of Abbreviations

***Ab*CM**	*A. butzleri* conditioned media
AIDS	Acquired immune deficiency syndrome
Arc	*Arcobacter* isolates of *Acanthamoeba*; the acanthamoebae that were isolated on *Arcobacter*
ASA.S1	*Acanthamoeba*-specific amplimer S1
BEs	Bacterial endosymbionts
BHI	Brain heart infusion
CL	Containment level
CNS	Central nervous system
Conc.	Concentration
CSF	Cerebrospinal fluid
DMSO	Dimethyl sulfoxide
Eco	*E. coli* isolates of *Acanthamoeba*; the acanthamoebae that were isolated on *E. coli*
EDTA	Ethylenediaminetetraacetic acid
Ent	*Enterococcus* isolates of *Acanthamoeba*; the acanthamoebae that were isolated on *Enterococcus*
FcR	Fc receptor
GAE	Granulomatous amoebic encephalitis
GFP	Green fluorescent protein
GI	Gastro-intestinal
GMOs	Genetically modified organisms
GTSA.B1	Genotype-specific amplimer B1
H&E	Hematoxylin-eosin
IGC	Intracellular growth co-efficient
LB	Luria Broth
MBP	Mannose binding protein
MOI	Multiplicity of infection
MSA	Multiple sequence alignment
NA	Nicotinic acid
NED	N-1-naphthylethylenediamine dihydrochloride
NS	Neff's saline
PBS	Phosphate buffer saline

PI	Pathogenicity island
PI	Pathogenicity island
PTKs	Protein tyrosine kinases
PTPs	Protein tyrosine phosphatases
PYG	Culture media for amoeba containing peptone, yeast extract and glucose
RCV	*Rhodococcus equi* containing vacuole
rpm	Revolutions per minute
SoV	Sodium orthovanadate
Sup VD-ED	*A. butzleri* ED-1 grown in VD media and filtered through 0.22 µm syringe filter
TSB	Tryptic soy broth
Vap	Virulence-associated protein
vapA	Virulence-associated protein A
vapC	Virulence-associated protein C
vapF	Virulence-associated protein F
VD	Vandamme media
WT	Wildtype
YCM	Yeast conditioned media

List of Definitions

Term	Definition
***Acanthamoeba* subgroups**	Intermediate sequences among a T type. These are described for T4 (T4-A, -B, -C, -D, -E, -F and -N). On the other hand T2/6 which are intermediate between T2 and T6 have three subgroups (T2/6-A, T2/6-B and T2/6-C).
***Acanthamoeba* subtypes**	The T4 subtypes of *Acanthamoeba* based on the sequence of DF3 fragment of 18S rRNA which is bound by the primer set 892 and JDP2 (~220 bp).
***Acanthamoeba* T types**	The genotyes of *Acanthamoeba* based upon 18S rRNA sequence analysis with at least 5% sequence diversity like. There are currently 18 T types (T1-T18) with two more suggested types (T19 and T20).
Adaptation	Change in an organism resulting from selection pressure.
AX2	Culture media for amoeba containing peptone, yeast extract (0.75%) and glucose in addition to sodium and potassium phosphate (see section 2.2.1. for details).
AX2+	Culture media for amoeba containing peptone, yeast extract (1.0%) and glucose in addition to sodium and potassium phosphate (see section 2.2.1. for details).
Character	Phenotypic traits possessed by an organism.
Commensalism	Symbiosis in which one organism benefits from the association, with other being neither harmed nor benefited.
Encyst	To enclose in a cyst in response to unfavourable circumstances.
Endosymbiont	An organism that lives inside the body of another organism establishing endosymbiosis.
Intracellular growth co-efficient (IGC)	A way of representing the relative intracellular growth pattern of two or more different bacteria at various time points during infection assay in terms of numbers which can be positive or negatives whereas a value of zero indicates the starting population of bacteria, a positive value indicates proliferation and a negative value indicates decrease in the number of bacteria. It is calculated by using the formula: IGC = (CFUs at a particular time point – CFUs at time zero) / CFUs at time zero. Since it is a relative value so IGC has no units.
Lytic	Ability to lyse the host cell, i.e., to rupture the host cell wall.
Mutualism	Symbiosis in which both organisms benefit from the association.

Parasite	An organism that benefits from the association with another organism while being harmful to the host.
Pathogenicity	Ability to cause disease. It is determined by its virulence factors.
Reservoirs (amoebae)	If bacteria are present in the amoebae as well as being able to multiply, the amoeba can be described as a reservoir.
Symbiosis	A phenomenon in which dissimilar organisms live together, i.e. association of two organisms throughout a significant portion of their life history.
Trojan horse	Literally, a strategy used to invade the town of Troy. Here, it refers to the protozoal "horse" that may bring a hidden amoeba-resistant microorganism within the human "Troy" protecting it from the first line of human defences.
Virulence	Degree of pathogenicity.
Virulence trait	Character that confers pathogenicity to an otherwise less pathogenic or non-pathogenic strain or organism.

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Chapter 1

Introduction

1. Introduction

Protists are eukaryotes with a unicellular level of organization, without cell differentiation into tissues (Abd *et al*., 2005) while protozoa are heterotrophic protists (Vaerewijck *et al*., 2014). Amoebae are protozoa with the ability to change shape by means of projections called pseudopodia and thus they exhibit amoeboid movement (Singleton and Sainsbury, 2006). Although there are many different types of free-living amoebae (FLA) four genera are important due to their pathogenic potential towards humans and animals namely *Acanthamoeba* (responsible for keratitis and granulomatous amoebic encephalitis), *Balamuthia* (cause skin & lungs infections and fatal encephalitis in children), *Sappinia* (few cases of brain infections) and *Naegleria* (non-opportunistic primary amoebic meningoencephalitis in children and adults) (Schuster and Visvesvara, 2004; Trabelsi *et al*., 2012; Visvesvara *et al*., 2007).

According to the very recent revised consensus classification (Ruggiero *et al*., 2015), which is a two-superkingdom, seven-kingdom scheme all the living organisms have been divided into two superkingdoms called Prokaryota and Eukaryota. The superkingdom Prokaryota is divided into five kingdoms (Animalia, Plantae, Fungi, Chromista and Protozoa). Two important phyla under Protozoa are Amoebozoa and Percolozoa. *Acanthamoeba* together with *Balamuthia* and *Sappinia* belong to the phylum Amoebozoa while *Naegleria* is under the phylum Percolozoa. The taxonomic position of *Acanthamoeba* along with *Balamuthia*, *Sappinia* and *Naegleria* is shown in Figure 1.1 (Khan, 2009; Ruggiero *et al*., 2015).

1.1. *Acanthamoeba*

Acanthamoeba were first described in 1930 (Castellani, 1930) and are among the most abundant and most prevalent protozoa in the world (Rodriguez-Zaragoza, 1994). With the discoveries of more and more *Acanthamoeba* species/genotypes (Corsaro and Venditti, 2010; Gast, 2001; Gast *et al*., 1996; Hewett *et al*., 2003; Horn *et al*., 1999; Łanocha *et al*., 2009; Magnet *et al*., 2014; Nuprasert *et al*., 2010; Pussard and Pons, 1977; Qvarnstrom *et al*., 2013; Stothard *et al*., 1998) there has been an ever growing increase in the interest towards studying various aspects of this versatile organism especially during the recent decades (Figure 1.2).

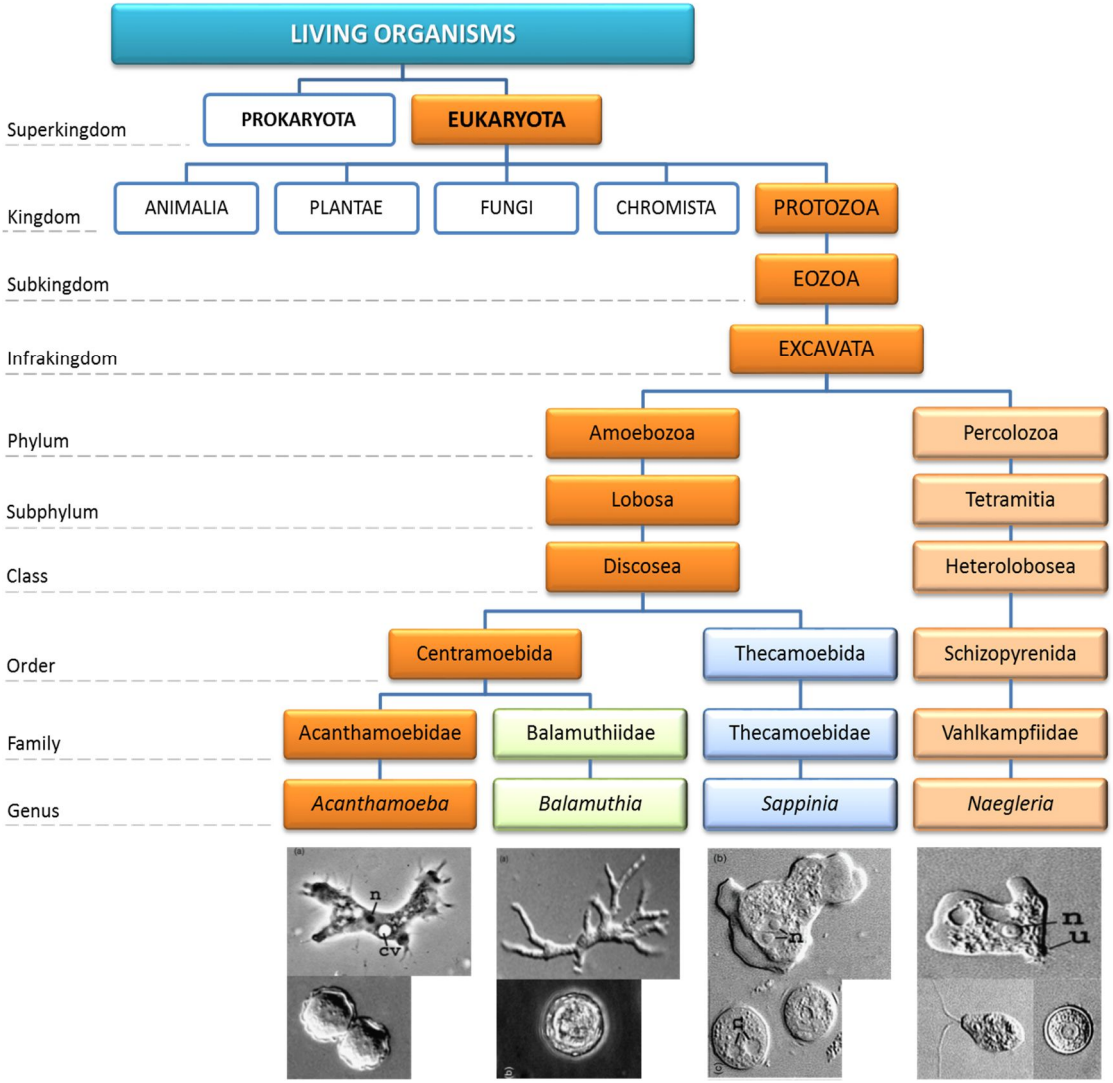


Figure 1.1: Taxonomic positions of FLA *Acanthamoeba* together with *Balamuthia*, *Sappinia* and *Naegleria* based on recent consensus classification scheme called Catalogue of Life (CoL) (Ruggiero *et al.*, 2015). Protozoa are one of the five kingdoms under the superkingdom Eukaryota. Three of these FLA belong to the same Phylum Amoebozoa while Naegleria belong to a different phylum Percolozoa. All these four FLA are associated with diseases in humans. The photographs represent from left to right *Acanthamoeba castellanii* (1000×) with trophozoite on the top and cyst on the bottom, *Balamuthia mandrillaris* (850×) with trophozoite on the top and cyst on the bottom, *Sappinia diploidea* (1000×) with trophozoite on the top and cyst on the bottom and *Naegleria fowleri* (1000×) showing trophozoites (top), cysts (bottom right) and in case of *N. fowleri* the flagellate form (bottom left). Photographs, taken from (Visvesvara *et al.*, 2007).

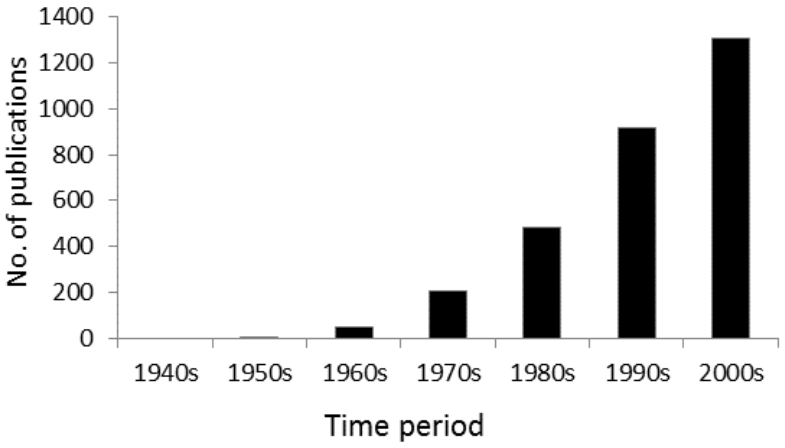


Figure 1.2: The decade-wise number of publications related to *Acanthamoeba* following their discovery in 1930 shows a constant increase in the interest in exploring various aspects of *Acanthamoeba*.

1.1.1. Structure

Acanthamoeba, under favourable conditions, exist as trophozoites which are single-celled vegetative forms (Figure 1.3-a) and can change their shapes because of pseudopodial movements. The concentration of water inside the cell is regulated by the contractile vacuole which is constantly engaged in expelling the extra water out of the cell. A number of food vacuoles are scattered throughout the cytoplasm that are formed as a result of ingestion of food material. Nucleus with its large nucleolus is a prominent structure.

The cell has an extensive network of endoplasmic reticulum with attached ribosomes. A number of mitochondria are also present in the cytoplasm. Numerous glycogen, lipid droplets and fibrils are also found in the cytoplasm. Actin and myosin form a network in the cell and together with other proteins play a primary role in the cellular movements (Khan, 2006). The cell membrane is distinct from that of mammalian cells due to presence of lipophosphonoglycan (Korn *et al*., 1974).

The cyst is a tough structure and is composed of two layers called ectocyst (outer layer) and endocyst (inner layer) that are separated by a variable interlayer space (Figure 1.3-b). The ectocyst is thicker than the endocyst, however, it is thinner at the operculum

(Figure 1.3-c). At the end of unfavourable conditions the cyst wall ruptures at the operculum releasing the trophozoites out (Bowers and Korn, 1969; Lemgruber *et al*., 2010). The cyst wall contains cellulose which makes up 10% of the dry weight of the cyst (Tomlinson and Jones, 1962).

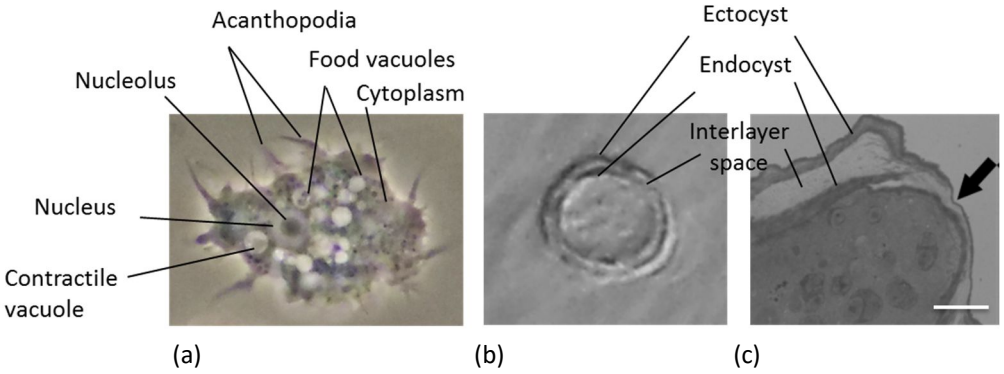


Figure 1.3: Structure of *Acanthamoeba* under microscope showing various components of (a) trophozoite (1000×), (b) cyst under light microscope (600×) and (c) cyst under transmission electron microscope showing operculum (arrow); scale bar = 2 mm (Figure c taken from Lemgruber *et al*. (2010)). Trophozoites are vegetative cells with no definite shape because of pseudopodial movements of cell. Acanthopodia are spike-like projections on the cell surface giving the organism its name (acanth=spike). The nucleus with prominent nucleolus is clearly visible in the cell along with many food vacuoles and a large contractile vacuole for expelling water of the cell. The cyst is a tough structure formed under unfavourable conditions with double cell walls; ectocyst and endocyst.

1.1.2. Genome

The genome size of *Acanthamoeba* has been estimated to be ~1×10^8 bp (Byers *et al*., 1990). The ploidy of *Acanthamoeba* is not agreed upon and is somewhere between 9 and 25 depending upon the stage of the cell cycle and the strain (Matsunaga *et al*., 1998; Rimm *et al*., 1988). The variation is probably due to destruction of nearly half of the DNA during encystment (Byers *et al*., 1991). *Acanthamoeba* bear 24 copies of 18S rRNA, therefore, each cell has as many as 600 copies of the 18S gene (Yang *et al*., 1994). Identification of *Acanthamoeba* at genus level is made through the presence of ASA.S1 (*Acanthamoeba*-specific amplimer S1) fragment which is highly specific to this genus (Schroeder *et al*., 2001).

1.1.3. Diversity in the environment

Acanthamoeba are widely distributed in the environment. The various sources where *Acanthamoeba* have been isolated from include soil, air, waters (bottled water, river, lakes, freshwater, stagnant, pond, seawater, swimming pools, public water supplies), ventilation ducts, air-conditioning units, sewage, compost, garden humus, sewage sediments, sewer sludge, harbour sediment, marine sediment, shellfish beds, plants roots, termite colony, various units of hospitals, surgical instruments, contact lenses, yeast culture, monkey kidney cell culture, human choriocarcinoma cells, and from humans (nasal cavities, lungs, skin lesions, cornea, CSF, brain tissues, faeces (Gordon *et al*., 1992; Harf and Monteil, 1989; Khan, 2003; Khan, 2006; Marciano-Cabral and Cabral, 2003; Ray and Hayes, 1954; Schuster and Visvesvara, 2004; Todd *et al*., 2015). The widespread presence of *Acanthamoeba* in the environment

1.1.4. Feeding

Acanthamoeba prey upon microorganisms including bacteria as well as yeast, algae and other protists (Allen and Dawidowicz, 1990; Weekers *et al*., 1993). The food is engulfed by phagocytosis as well as pinocytosis (Allen and Dawidowicz, 1990; Alsam *et al*., 2005; Bowers and Olszewski, 1972). Phagocytosis is a direct ingestion of food particle achieved by wrapping it in the form of a vacuole while pinocytosis is used for ingestion of molecules which are taken up by invagination of cell membrane (Bowers and Olszewski, 1972). Both of these mechanisms are not only different but also independent of each other (Chambers and Thompson, 1976).

However, the choice of either of these mechanisms, and whether the pathogenic and non-pathogenic *Acanthamoeba* have any difference in this regard are unclear (Alsam *et al*., 2005). It is interesting that following internalization of a particle within a vacuole, *Acanthamoeba* can differentiate between the digestible and non-digestible content; the former is digested while the latter is expelled from the cell (Bowers and Olszewski, 1983).

1.1.5. Life cycle

Acanthamoeba have a dual life style comprised of a vegetative trophozoite form with the potential to divide by binary fission under normal conditions, and a metabolically inactive but highly resistant cyst formed under unfavourable circumstances (desiccation, starvation, anoxia, osmolarity, extreme pH, temperature and others) which can last until the return of favourable conditions (Aksozek *et al*., 2002; Lloyd *et al*., 2001). Although trophozoites are the infective forms cysts can also gain entry into body through eye causing *Acanthamoeba* keratitis (AK) and through nasal passages/skin causing granulomatous amoebic encephalitis (GAE) (Figure 1.4).

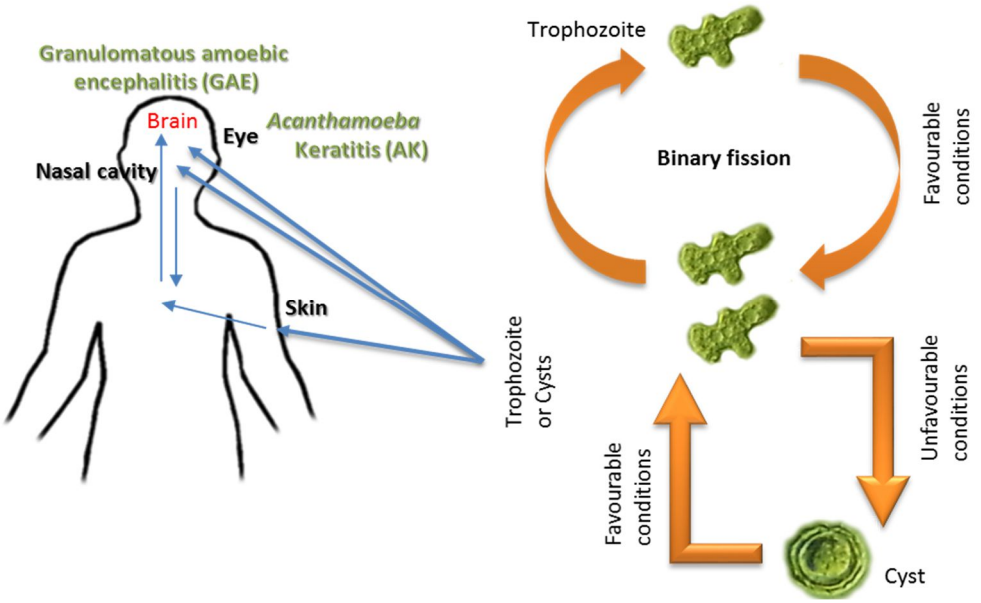
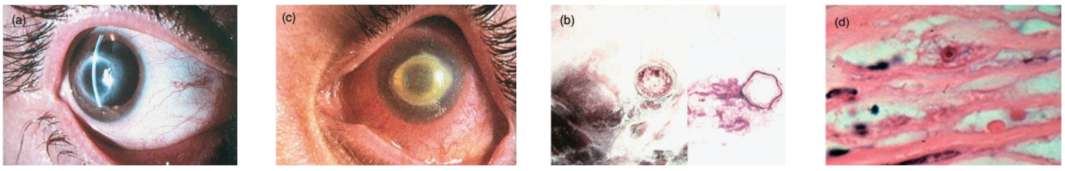


Figure 1.4: Diagrammatic representation of the life cycle of *Acanthamoeba*. In addition to living freely in the environment, *Acanthamoeba* also found as opportunistic pathogens in humans. Under normal conditions *Acanthamoeba* are found as trophozoites which is the infective form. Trophozoites divide by mitosis (binary fission). Under unfavourable conditions, trophozoites transform into highly resistant cysts having a thick wall. The cyst form persists until the return of favourable conditions when the cyst wall ruptures (a process excystment) and the trophozoite emerges out. Cysts can also be directly infective; by gaining entry into the host body. Various portals of entry into the body include eye, nasal passages or skin. Infection of eye is manifested in the form of keratitis while entry through nasal and dermatological routes is followed by transportation of *Acanthamoeba* through blood circulation to brain, causing GAE. In immunocompromised individuals, *Acanthamoeba* can disseminate from brain to lungs and skin where they cause infection of these organs.

1.1.6. Diseases caused by *Acanthamoeba*

1.1.6.1. *Acanthamoeba* keratitis (AK)

Acanthamoeba keratitis (AK) has been recognized since mid-1970's (Jones *et al*., 1975; Naginton *et al*., 1974). Although rare. AK is a painful infection that can be serious enough to cause blindness in untreated cases. It is characterized by inflammation of the cornea resulting in photophobia, a characteristic 360° or paracentral stromal ring infiltrate and corneal lesion which is not treatable with routine antibiotics (Figure 1.5-a,b). Usually one eye is affected but both eyes can also be involved (Visvesvara *et al*., 2007). The presence of *Acanthamoeba* trophozoites and cysts can be seen in corneal scrapings and tissue (Figure 1.5-c,d). Although contact lens wearers are the main and high-risk group of people likely to have AK infections (Beattie *et al*., 2003; Tomlinson *et al*., 2000) cases have also been seen as a result of physical damage to the eye (Sharma *et al*., 1990) and the cytotoxicity caused by the release of cysteine and serine proteases (Lorenzo-Morales *et al*., 2005).



(a) (b) (c) (d)

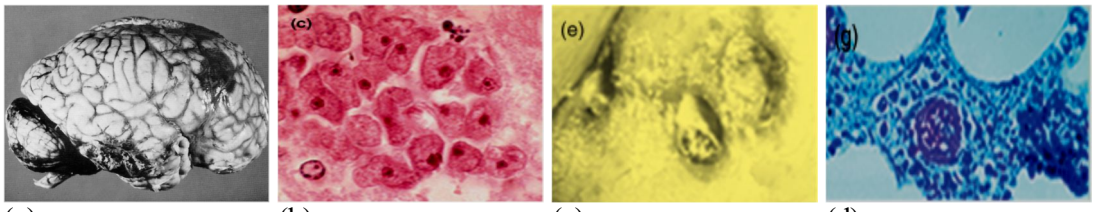
Figure 1.5: The damages caused by *Acanthamoeba* keratitis (AK). (a) A 360° infiltrate which is characteristic of AK is seen on slit-lamp examination of an affected patient. (b) Perforation of the cornea. (c) *Acanthamoeba* trophozoite and a cyst can be seen in a corneal scraping (×1000). (d) The corneal tissue from infected eye showing a trophozoite, stained with H&E (×1000). Taken from (Visvesvara *et al*., 2007).

1.1.6.2. Granulomatous amoebic encephalitis (GAE)

Granulomatous amoebic encephalitis (GAE), recognized in early 1970's (Jager and Stamm, 1972), is a highly pathogenic disease with nearly 100% mortality and is characterized by fever, nausea, vomiting, headache, intracranial pressures, seizures with eventual death (Khan, 2007). The disease usually causes disseminated infection in the body affecting lungs and causing skin lesions especially in immunocompromised patients (Aichelburg *et al*., 2008; Sison *et al*., 1995).

Acanthamoeba are believed to be transmitted to the brain through the nasal route making their way down to the lungs from where they are transferred to the brain through circulation or olfactory neuroepithelium. Amoebae that gain entry into the human body through skin can also gain entry into the brain through blood circulation. Beaching of the main hurdle, the blood-brain barrier, is achieved using either of the two strategies *viz* contact-dependent and contact-independent mechanism. The former involves apoptosis of the endothelial cells while the latter employs amoebic proteases for destruction of the extracellular matrix.

Once the amoebae break the barrier they start causing actual damage to the brain (Massilamany and Reddy, 2011) which can be seen on autopsy as the isolated areas of congestion along with cerebral oedema, haemorrhages and necrotic areas (Figure 1.6-a). Amoebic trophozoites, and cysts are clearly seen in histological sections (Figure 1.6-b). Cases have also been reported with only skin ulcer lesions (Figure 1.6-c) but without the dissemination of disease to the nervous system (Slater *et al*., 1994; Visvesvara *et al*., 1990). The cysts and trophozoites of *Acanthamoeba* can be seen in skin lesions (Figure 1.6-d) nervous tissues, lung parenchyma and prostate.



(a) (b) (c) (d)

Figure 1.6: Pathological features of GAE affected brain and skin infections caused by *Acanthamoeba*. (a) Lateral view of a human brain showing areas of congestion and haemorrhages. (b) A histological stained section of cortex region showing numerous *Acanthamoeba* trophozoites (×1000). (c) Ulcerative lesions in the skin of an immunocompromised patient. (d) An H&E-stained section of the skin ulcer showing *Acanthamoeba* cysts and trophozoites. Figure (a) is taken from (Martinez, 1985) while (b), (c) and (d) are from (Visvesvara *et al*., 2007).

1.1.7. Isolation from soil

Isolation of *Acanthamoeba* from soil is usually made by using *E. coli* as the monoxenic source of food (Chung *et al*., 1996; Ertabaklar *et al*., 2007; Kong *et al*., 1995; Lorenzo-Morales *et al*., 2005; Maciver *et al*., 2013; Rahdar *et al*., 2012; Reyes-Batlle *et al*.,

2014; Rezaeian *et al*., 2008; Tsvetkova *et al*., 2004). Bacteria are overlaid on to the surface of non-nutrient agar plate for this purpose. Purification is achieved by repeatedly passaging *Acanthamoeba* on bacterial agar plates. This is then followed by adaptation to liquid culture (axenization) which may take considerably long periods of time (Zanella *et al*., 2012).

1.1.8. Classification of *Acanthamoeba*

Members of the genus *Acanthamoeba* have a distinct appearance so they are easily recognized especially in the cyst form. However, the identification to species level has been challenging (Pussard and Pons, 1977) ever since their discovery in 1930 by Castellanii in cultures of the yeast *Cryptococcus pararoseus* (Castellani, 1930). Various attempts were made from time to time to establish a robust classification system for *Acanthamoeba*. Figure 1.7 outlines main events in the classification of *Acanthamoeba* in chronological order from their discovery until recent advancements. *Acanthamoeba* was initially placed in the genus *Hartmannella* and thus named *Hartmannella castellanii* (Castellani, 1930) and (Douglas, 1930).

A year later it was realised on close inspection that the genus *Hartmannella* was actually a group of different types of amoebas with different characteristics. Therefore, the genus *Hartmannella* was split apart into three genera named *Hartmannella* (possessed round, smooth-walled cysts with cylindrical spindle), *Glaeseria* (manifested nuclear division in the cyst) and *Acanthamoeba* (cysts were double-walled with ostioles while the outer layer was irregular, also manifested pointed spindles during mitosis) (Volkonsky, 1931).

Singh (1952) considered the classification of amoebas on the basis of morphological characteristics and the mitotic spindle shape of no significance. Therefore, they discarded the genus *Acanthamoeba*. However, later Pussard (1966) disagreed with Singh (1952) and found cyst morphology useful characteristic at generic level and recognised the genus *Acanthamoeba* once again, however, he did agree that spindle shape was of no use. Page (1967) also supported the finding of Pussard (1966) that the spindle shape was not a reliable characteristic and considered that cyst morphology

and presence of acanthopodia sufficient. Therefore, he declared the *Hartmannella* and *Acanthamoeba* correct.

In 1977, Pussard and Pond (1977) came up with a revolutionary three-group classification of *Acanthamoeba* at subgenus level based on cyst morphology. They recognised a total of 18 different species of *Acanthamoeba*. Despite the limitations and introduction of more advanced methods used later for subgenus classification of *Acanthamoeba*, the morphological classification is still used. Stratford and Griffiths (1978) reported that cyst morphology may not always be trusted as it can change with varying environmental conditions.

Various other possible techniques were then probed for reliable classification of *Acanthamoeba* but with varying success and none could gain universal acceptance except the T typing system (discussed later in this chapter). These included isoenzyme patterns (De Jonckheere, 1983), restriction fragment length polymorphism (RFLP), (Bogler *et al*., 1983b), fatty acids profile (Costas and Griffiths, 1984), ribotyping, (Chung *et al*., 1998) and 16S mitochondirial sequence analysis (Ledee *et al*., 2003).

In 1996, the classification of *Acanthamoeba* was made using an alternative approach by using 18S rRNA sequence. Initially four different genotypes (called T types) were identified that had >5% sequence dissimilarity and were called T1 to T4 (Gast *et al*., 1996). T types upto T19 have been reported (Corsaro and Venditti, 2010; Hewett *et al*., 2003; Horn *et al*., 1999; Łanocha *et al*., 2009; Magnet *et al*., 2014; Nuprasert *et al*., 2010; Qvarnstrom *et al*., 2013; Stothard *et al*., 1998) while T20 has very recently been also claimed (Fuerst *et al*., 2015).

*T20 has recently been suggested by Fruest (2015).

Figure 1.7: Key chronological events in the classification of *Acanthamoeba* since first discovery in 1930. Numbers (1-24) in the circles correspond to the references as: 1=Castellani (1930) and Douglas (1930); 2=Volkonsky (1931); 3=Singh (1952); 4=Pussard (1966); 5=Page (1967); 6=Pussard and Pons (1977); 7=Stratford and Griffiths (1978); 8=De Jonckheere (1983); 9=Bogler *et al*. (1983); 10=Costas and Griffiths (1984); 11=Costas and Griffiths (1986); 12=Page (1988); 13=Johnson *et al*. (1990); 14=Gast *et al*. (1996); 15=Stothard *et al*. (1998); 16=Chung *et al*. (1998); 17=Horn *et al*. (1999); 18=Gast *et al*. (2001); 19=Hewett *et al*. (2003); 20=Ledee *et al*. (2003); 21=Corsaro and Venditti (2010); 22=Nuprasert *et al*. (2010); 23=Qvarnstrom *et al*. (2013); 24=Magnet *et al*. (2014).

1.1.8.1. Morphological classification of *Acanthamoeba*

The big event in the classification of *Acanthamoeba* at sub-genus level was observed with the proposed division of all *Acanthamoeba* species (18 at that time) into three groups (I, II and III) based on the morphological characteristics of the cyst. This classification system for *Acanthamoeba* was taken up by others and formed the basis of Page's key (Page, 1988). The key features of the three morphological groups are listed in Table 1.1.

Table 1.1: Differential key characteristics of cysts belonging morphological groups I, II and III of *Acanthamoeba* (Pussard and Pons, 1977).

Group I	Group II	Group III
• Large cysts with average cyst diameter of ≥18 µm. • Ectocyst is rounded and smooth or gently wrinkled. • Endocyst is more or less stellate. • Ectocyst and the endocyst are widely apart. • Endocyst fuses with ectocyst at the ends of arms or rays.	• Average cyst diameter is usually <18 µm. • Ectocyst (which may be thick or thin) is usually wrinkled or mammilated. • Endocyst can be stellate, polygonal, triangular, round or oval and usually with no well-developed arms or rays. • Ectocyst and the endocyst are variable and either very close or wide apart.	• Average cyst diameter is usually <18 µm. • Ectocyst is thin and may be gently rippled or un-rippled. • Endocyst is usually round and may have 3-5 gentle corners.

The species assigned to the morphological groups I, II and III of *Acanthamoeba* along with the photographs of the representative cyst are shown in Figure 1.8. This system of classification gained popularity, however, with the growing number of species of *Acanthamoeba*, identification based on morphological characteristics alone proved insufficient as manifested by the variations in the cyst morphology within the same strain due to changing growth conditions such as ionic strength (Sawyer, 1971). Similar findings were reported for *A. castellanii* (Stratford and Griffiths, 1978). A second flaw with this classification system was poor correlation of a certain species with its pathogenic potential, for examples, *A. castellanii* includes strains with different pathogenesis ranging from virulent to non-virulent. Therefore, it was realized that other features should have been considered and not just morphology.

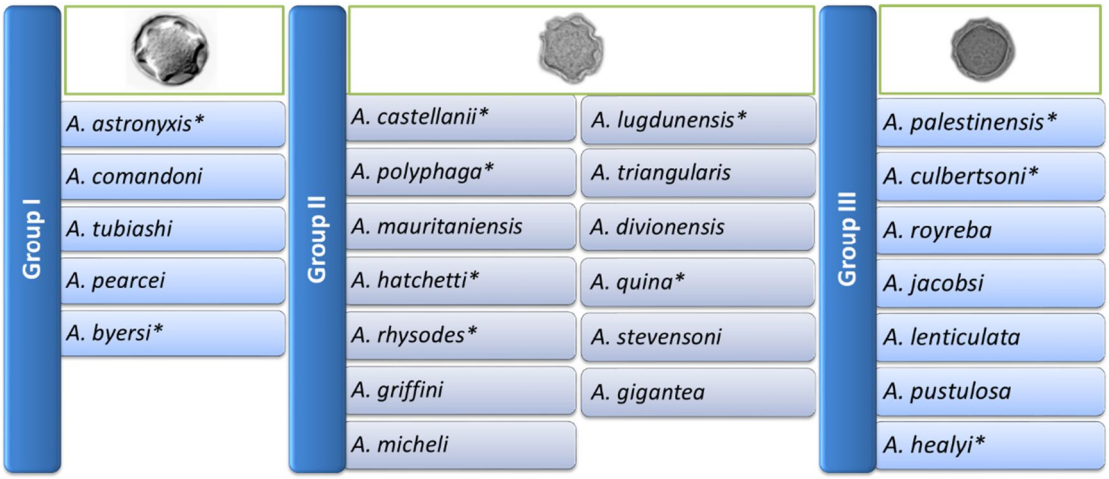


Figure 1.8: Species of *Acanthamoeba* assigned to one of the three morphological groups (I, II and III). The pathogenic species are indicated by asterix (*). The photographs are taken from Qvarnstrom *et al*. (2013), group I; Corsaro *et al*. (2015), group II; Gianinazzi *et al*. (2010), group III.

1.1.8.2. Attempts for non-morphological classification of *Acanthamoeba*

Various attempts have previously been made to classify *Acanthamoeba* successfully at subgenus level using non-morphological methods as described below:

Costas and Griffiths (1984) investigated the taxonomic significance of **fatty acid profile** for *Acanthamoeba*. They studied the fatty acid composition of six different species of *Acanthamoeba* comprising a total of 11 strains. These species included *A. castellanii, A. polyphaga, A. rhysodes, A. astronyxis, A. griffini* and *A. palestinensis*. Unfortunately, the results were very disappointing because there were significant variations among the fatty acid profiles not only between the batches but also at different temperatures. The results were so overlapping that it was impossible to assign any pattern to any species or strain.

Isoenzyme analysis was used by a number of workers (Chung *et al*., 1996; Costas and Griffiths, 1985; Daggett *et al*., 1985; De Jonckheere, 1983; Kong *et al*., 1995; Visvesvara *et al*., 1983; Weekers and De Jonckheere, 1997) as a possible alternate and reliable approach for species classification of *Acanthamoeba*, however, intraspecific heterogeneity of the zymograms has been observed for several kinds of isoenzymes. It was stressed that although isoenzyme analysis may be a good tool for species

classification of *Acanthamoeba*, but since different band pattern can be obtained even within the same species, it must not be used as a sole tool for species identification of new isolates. Isoenzyme patterns in most of the cases did not agree with the species names and, therefore, this technique failed to became a standard method for classification of *Acanthamoeba*. Different isoenzyme patterns with changing growth conditions is a serious issue in considering this technique as a standard for classification of *Acanthamoeba* (Weekers and De Jonckheere, 1997).

Restriction fragment length polymorphism (RFLP) analysis is a DNA profiling technique which involves digestion of a DNA fragment by using restriction enzymes. The resulting smaller fragments when separated using gel electrophoresis, give rise to a certain pattern of bands which can then be compared with the RFLP pattern of DNA from isolates. RFLP was used by many workers (Bogler *et al*., 1983a; Kanno *et al*., 1998; Kong *et al*., 2002; Liu *et al*., 2006; Xuan *et al*., 2008; Yagita, 1993; Yagita and Endo, 1990; Yu *et al*., 2004). However, due to profound inter-strain diversity it was concluded that this technique could be used for strain identification, differentiation, and characterization only but not for species identification (Chung *et al*., 1996; Kong *et al*., 1995; Kong *et al*., 2002).

Mitochondrial 16S-like rRNA sequence analysis was considered a good target due to the lack of introns in their sequences with only ~1500 bp length compared to ~2300 bp for 18S rRNA (Kong, 2009; Ledee *et al*., 2003; Liang *et al*., 2010; Rahman *et al*., 2013; Xuan *et al*., 2008). Both 18S and 16S-like sequence typing of *Acanthamoeba* were found useful for species identification and also considered an additive tool for better understanding of molecular taxonomy of *Acanthamoeba* although both these regions possess different and unique properties (Rahman *et al*., 2013).

Riboprinting which combines the power of PCR and digestion by restriction endonucleases to generate specific patterns was found not only comparable to 18S rRNA sequencing (Kong and Chung, 2002; Yu *et al*., 1999) but also it was considered as a cheaper, simpler (Clark and Diamond, 1997) and a useful technique for classification of *Acanthamoeba* (Chung *et al*., 1998), however, the inability to

distinguish between some species like *A. castellanii* and *A. polyphaga* remains a concern (Kong and Chung, 1996). A new classification scheme for *Acanthamoeba* was suggested by (Gast *et al*., 1996) based on 18S rRNA gene sequences called as T typing.

1.1.8.3. T Typing system (classification scheme based on 18S rRNA sequences)

A "T type" is a genotype of *Acanthamoeba* based on its 18s rRNA sequence and the sequence diversity is the basis for various T types. It was introduced by Gast *et al*. (1996) as a molecular approach to overcome the limitations of *Acanthamoeba* subgenus classification usually encountered with morphological and other approaches. Initially, four distinct genotype clusters were identified based on complete sequences of nuclear small ribosomal subunit RNA genes *(Rns)* by studying 18 strains, that were named as T1, T2, T3 and T4 (Gast *et al*., 1996). The *Acanthamoeba* genotype was defined as the whole group of *Acanthamoeba* strains that differed from one another by <6% based on 18S rRNA sequences but this was later reduced to <5% (Stothard *et al*., 1998) and even further reduction has been suggested down to <4% recently (Fuerst, 2014).

New T types of *Acanthamoeba* are claimed on the basis of full-length 18S rRNA sequence, however, T15 and T16_A are exceptions. In case of T15, GTSA.B1 (genotype-specific amplimer B1) fragment which is ~1500 bp long, was used (Hewett *et al*. 2003). Similarly in case of T16_A, the Ami fragment, which is ~830 bp long, was used (Łanocha *et al*., 2009). One of the T types was wrongly assigned as T14 (Horn *et al*. 1999) as it did not differ >5% from previously reported sequences but later this was pointed out and was assigned as T13 by Gast (2001) who also reported the the actual T14. By 2014, 19 T types were recognized (Table 1.2) while T20 has been suggested from a previously reported sequence (Fuerst *et al*., 2014). Species association with T types is not always strict which means that a particular T type can contain different species (like T4 has *A. castellanii, A. polyphaga, A. lugdunensis, A. rhysodes*) or same species can belong to different T types (like *A. castellanii* which belongs to T1 as well as T4).

Table 1.2: An account of the *Acanthamoeba* T types (T1 to T19) in chronological order with relation to species (if any) and the source and location of sample. The species name association with T types is not always strict.

Year	T type	Species association (if any)	Source of isolation (location)	Reference
1996	T1	*A. castellanii*	Brain (USA)	Gast *et al*. (1996); Stothard *et al*. (1998)
	T2	*A. palestinensis, A. pustulosa, A. polyphaga*	Soil (Israel)	
	T3	*A. griffin, A. polyphaga, A. peurcei*	Beach bottom (USA)	
	T4	*A. castellanii, A. polyphaga, A. lugdunensis, A. rhysodes, A. hatchetti, A. culbertsoni Acanthamoeba* sp.	Yeast culture (UK) Keratitis (USA, India) Soil (USA, Japan, UK) Lung infection (USA)	
1998	T5	*A. lenticulata*	Swimming pool (France) Sewage plant (USA) Nasal mucosa (Germany) Freshwater stream (USA) Water sample (France) Sewage dump site (USA)	Strothard *et al*. (1998)
	T6	*A. palestinensis*	Swimming pool (France)	
	T7	*A. astronyxis*	Lab water (USA)	
	T8	*A. tubiashi*	Freshwater (USA)	
	T9	*A. comandoni*	Soil (France)	
	T10	*A. culbertsoni*	Human cell culture (USA)	
	T11	*A. stevensoni*	Marine sediment (USA)	
		A. hatchetti	Brackish water (USA)	
	T12	*A. healyi*	GAE, brain (Barbados)	
1999	T13	*Acanthamoeba* sp.	Contact lens (USA)	Horn *et al*. (1999)
2001	T14	*Acanthamoeba* sp.	Eye (Pakistan)	Gast (2001)
2003	T15	*A. jacobsi*	Waters (USA, Australia, UK)	Hewett *et al*. (2003)
2009	T16_A	*Acanthamoeba* sp.	Bronchoaspirate fluid (Poland)	Lanocha *et al*. (2009)
2010	T16	*Acanthamoeba* sp.	Freshwater pond (South Italy)	Corsaro and Venditti (2010)
		Acanthamoeba sp.	Freshwater pond (South Italy)	
2010	T17	*Acanthamoeba* sp.	Lampangpui pond (Bangkok)	Nuprasert *et al*. (2010)
		Acanthamoeba sp.	Freshwater pond (Bangkok)	
2013	T18	*A. byersi*	Skin and brain (USA)	Qvarnstrom *et al*. (2013)
2014	T19	*Acanthamoeba* sp.	Waste water (Spain)	Magnet *et al*. (2014)

*T20 has recently been suggested by Fruest (2015).

Based on the accumulated data in the gene banks until recently, T4 has been found as the major T type (Figure 1.9) (Fuerst, 2014). T4 is not only abundant in the environment but also is the most abundant among the clinical isolates (Maciver *et al*., 2013). The T1 to T19 T types of *Acanthamoeba* have been isolated from a variety of environments. Further investigations are required to understand why many of the T types are so limited and not widely distributed which might be a consequence of their association with a particular environmental factor such as abundance of prey bacteria.

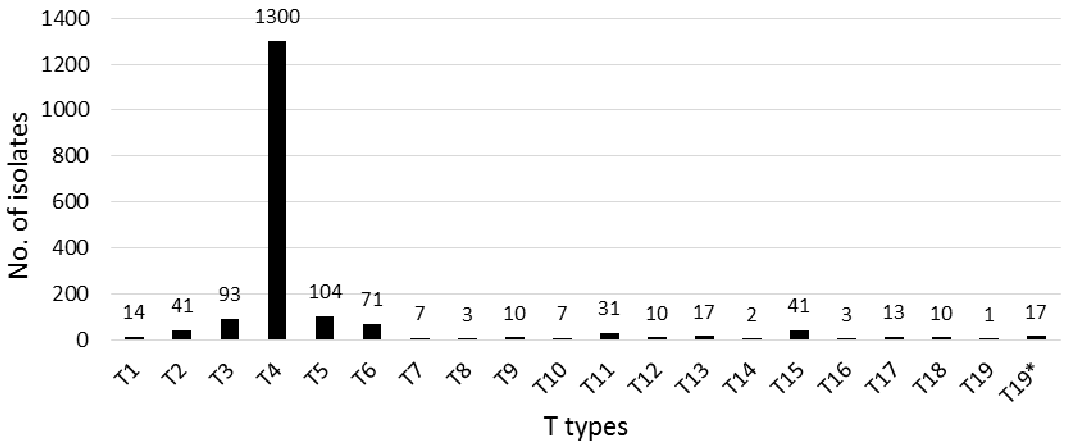


Figure 1.9: The number of *Acanthamoeba* isolates reported for each of the 19 genotypes based on 18S rRNA gene sequence as per data submitted to the international DNA databases (total 1794 sequences). T4 is the most abundant T type while T19 is the rarest. There is conflict in the designation of T19 (Magnet *et al*., 2014) and T19* (Fuerst *et al*., 2014). Data taken from Fuerst (2014).

Despite the wide acceptance of The T typing system with more than 1700 typed isolates of *Acanthamoeba* have been deposited in Genbank, there still remain limitations associated with this method. Some scientists still use morphological grouping or use species names which arises confusion. The introduction of new genotypes that are less than 5% divergent from the previously reported genotypes is another challenge (Crary *et al*., 2012). The 18S rRNA gene has secondary structures and introns. The variations in the sequences of different genotypes of *Acanthamoeba* are great which result in gaps and present difficulty during alignment of sequences and thereby phylogenetic inference.

2.1.8. Interaction of *Acanthamoeba* with bacteria

The interaction between amoebae and bacteria is dynamic and important where not only amoebae but bacteria are also benefited. This can be of much more significance in case of human pathogens capable of surviving in amoebae (Thomas *et al*., 2010). *Acanthamoeba*-bacteria interaction was originally considered as merely predator-prey relation where bacteria served as the food (Weekers *et al*., 1993a) and thus *Acanthamoeba* were considered only to play a role in regulating the bacterial populations in the environment (Ronn *et al*., 2002). However, it was gradually learnt that the relationship was not so simple and it could become symbiotic resulting in enhanced bacterial survival and thus dissemination in the environment (Laskowski-

Arce and Orth, 2008). Similarly bacteria were found to influence the survival of *Acanthamoeba* because of preferential feeding on different kind of bacteria (Weekers *et al*., 1993). An overview of the possible outcomes of *Acanthamoeba*-bacteria interaction is sketched in Figure 1.10.

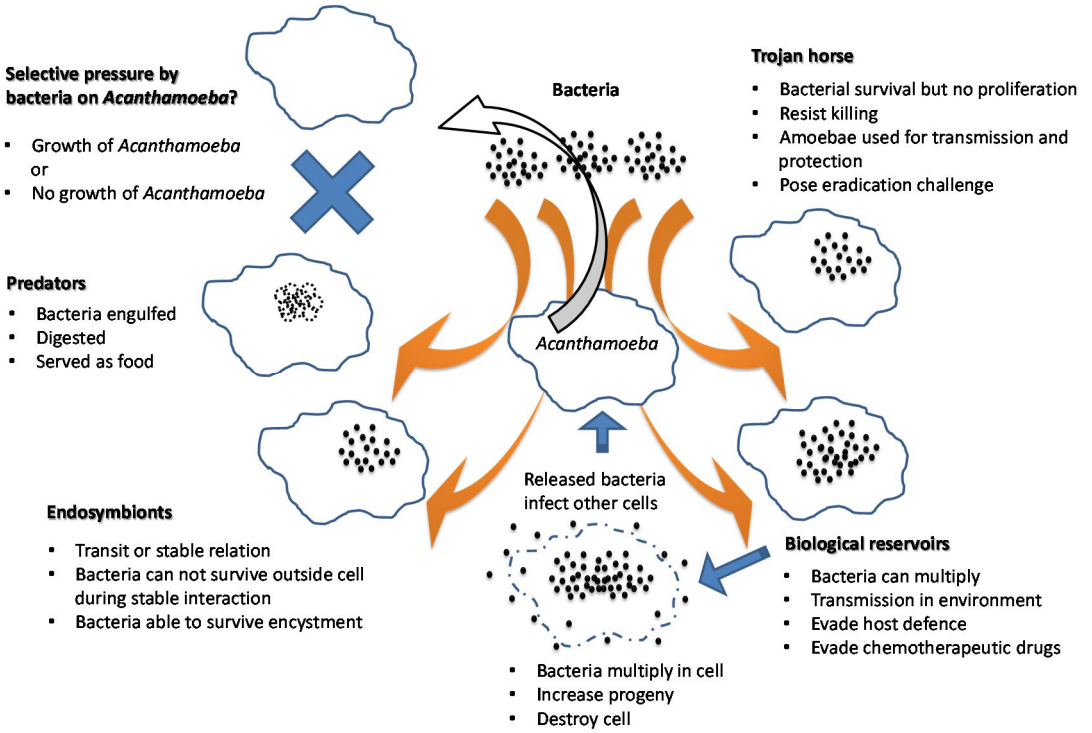


Figure 1.10: Various levels of interaction *Acanthamoeba* can have with bacteria. As originally believed, the interaction was limited to predation whereby *Acanthamoeba* use bacteria merely as food. However, *Acanthamoeba* have a variety of other ways of interacting with bacteria including their role either as Trojan horses where bacteria seem to be of greater advantage in using *Acanthamoeba* as shelter and carrier; or as endosymbionts which in strict terms is obligate in nature where bacteria cannot survive outside *Acanthamoeba*; or the very aggressive where bacteria bypass defensive mechanisms of *Acanthamoeba* and after multiplication lyse the cell releasing bacteria.

1.1.9.1. Effect of bacteria on *Acanthamoeba* diversity

Both *Acanthamoeba* and bacteria influence each other during their interaction. It has already been reported that bacteria preferentially feed different types of bacteria. Studies carried out in this regard by Weekers *et al*., (1993) indicated that non-pigmented bacteria *E. coli* K-12 and *Klebsiella aerogenes* served as better food for *Acanthamoeba* but with pigmented bacteria (*Chromatium vinosum* and *Serratia marcescens*) almost no growth was observed while low to moderate growth was

observed in indigenous soil bacteria (*A. tumefaciens, A. simplex, B. megaterium, B. subtilis, M. luteus* and *P. fluorescens*). Similarly, Schuster, (1993) studied a number of bacteria and according to preference of feeding, divided them into different groups ranked as readily eaten (*B. cereus* and *E. cloacae*), moderately consumed (*S. aureus*), poorly consumed (*S. marcescens*), and inedible (*P. aeruginosa* and *C. violaceum*).

However, if we look at this in the perspective when only a single type of bacterium is available as food as it happens during isolation of *Acanthamoeba* from environmental or clinical samples, in which case mostly only *E. coli* is used as monoxenic source of food, the question that arises is "can a single bacterium like *E. coli* effectively allow isolation of every genotype of *Acanthamoeba* in the sample?" This is an important question that has never been addressed by linking the effect of different bacteria as a food source with the recovery of genotypes of *Acanthamoeba*.

As the genotyping data for *Acanthamoeba* collected so far shows that around the globe 19 genotypes of *Acanthamoeba* exist (T1-T19) with T4 taking the major share (Fuerst, 2014; Maciver *et al*., 2013) accounting for more than 90% of all the T types while surprisingly at the same time there are few genotypes that have rarely been encountered such as T19, T14, T8, T16, T7, T10, T9, T12 and T18 all of which have been found just 10 or less times each (Fuerst, 2014; Magnet *et al*., 2014). The scarcity of few genotypes can either be a genuine figure or it may be because same kind of bacteria (*E. coli*) have been used universally for the isolation of *Acanthamoeba*. Can other T types or subtypes can be recovered if different bacteria are used is yet to be addressed.

1.1.9.2. Effect of *Acanthamoeba* on bacteria

1.1.9.2.1. *Acanthamoeba* as predators

The primary role of free living amoebae (FLA) including *Acanthamoeba* in the environment is that of predator (feeding bacteria) and in doing so they eliminate a major population of these bacteria which is ecologically significant for maintaining their number to an optimal level in the environment as well as for nutrient cycling (Rosenberg *et al*., 2009). It is important that predation of bacteria by the

Acanthamoeba is applicable to only those bacteria that don't resist the intracellular killing and merely serve as food for *Acanthamoeba* (Khan and Siddiqui, 2014). In addition to these bacteria, other bacteria can resist killing inside the cell. Such bacteria have two fates; either they just avoid killing by *Acanthamoeba* but don't multiply, or they also they not only avoid killing but also have the potential to multiply and thereby *Acanthamoeba* in this case act as biological reservoirs (Greub and Raoult, 2004).

1.1.9.2.2. *Acanthamoeba* as "Trojan horses"

This term is used to indicate the presence of bacteria residing inside *Acanthamoeba*, however, it is distinct from the carrier state which is merely attachment of bacteria on the cell surface. The "Trojan horse" behaviour of bacteria within *Acanthamoeba* in the form of 'just survival' but no proliferation appears more like forced entry of bacteria into the *Acanthamoeba* cells for protection from the unfavourable circumstances or use them for transmission to new places. *Acanthamoeba* can, therefore, act as vectors for transmission of bacteria to humans (Barker and Brown, 1994; Bozue and Johnson, 1996). Moreover, because of their remarkable ability to withstand chlorine, *Acanthamoeba* provide hideouts to internalized bacteria, making their eradication from the water challenging (Khan and Siddiqui, 2014; King *et al*., 1988).

1.1.9.2.3. *Acanthamoeba* as biological reservoirs

This important mode of interaction between *Acanthamoeba* and bacteria is reserved for pathogenic bacteria who are capable of modulating the intracellular trafficking pathways of the amoebae and thus by bypassing the killing mechanisms they can also proliferate intracellularly. The role of *Acanthamoeba* in acting as biological reservoirs for bacterial pathogens is of great significance (Greub and Raoult, 2004) and is a concern in case of human pathogens to whom *Acanthamoeba* not only provide safe heavens (by allowing them to proliferate and cordon them off from the host immune system/drug action) but also they transmit the bacteria through the environment (Khan and Siddiqui, 2014).

Attention was paid to the significance of this interaction when it was found that *Legionella pneumophila* were able to survive and even multiply in *Acanthamoeba*

(Rowbotham, 1980). Since then a wide range of other bacterial pathogens have been studied and reported to survive or proliferate in *Acanthamoeba* including human pathogens such as *Mycobacterium* sp. (Adekambi *et al*., 2006; Cirillo *et al*., 1997), *Listeria monocytogenes* (Ly and Muller, 1990), *Chlamydophila pneumoniae* (Horn *et al*., 2000), *E. coli* O157 (Barker *et al*., 1999), *Burkholderia cepacia* (Marolda *et al*., 1999), *Helicobacter pylori* (Winiecka-Krusnell *et al*., 2002), *Shigella sonnei* (Jeong *et al*., 2007) and *Vibrio cholera* (Abd *et al*., 2010).

The breakthrough finding emerged when it was first observed that the intracellular survival in *Acanthamoeba* could significantly enhance the virulence of *L. pneumophila* (Cirillo *et al*., 1999). Such interaction has also been linked to enhanced antibiotic resistance (Barker *et al*., 1999). *Mobiluncus curtisii* which is an obligate anaerobe bacterium was found to be able to persist even in an aerobic environment after survival in *Acanthamoeba* (Tomov *et al*., 1999). Therefore, amoebae emerged as environmental sources, reservoirs and transmission vehicles for bacterial pathogens.

The implication of such interactions in the broader perspective is that whether *Acanthamoeba* can serve as environmental reservoirs that protect and disseminate the pathogens; and/or the bacterial pathogenicity can be modulated as a result of intracellular survival which may pose a serious threat to human health, necessitating the investigation of this important amoeba-bacteria relation (Khan and Siddiqui, 2014).

1.1.9.2.4. Bacterial endosymbionts of *Acanthamoeba*

Bacterial pathogens can establish short-term or long-term endosymbiotic relations with *Acanthamoeba* (Schmitz-Esser *et al*., 2008). Long-term associations are more important and have been described since 1970 (Proca-Ciobanu *et al*., 1975). However, their detailed identification had been unclear until the use of modern techniques. Bacterial endosymbionts (BEs) of *Acanthamoeba* belong to either of the four main evolutionary lineages within the domain *Bacteria* which include *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*, and *Chlamydiae* (Amann *et al*., 1997; Birtles *et al*., 2000; Horn *et al*., 1999; Horn *et al*., 2002; Horn *et al*., 2001; Horn *et al*., 2000).

It is interesting that a single endosymbiont can be found distributed to distant parts of the world but the simultaneous existence of different endosymbionts is usually not observed which indicates presence of a specific strategy required for building up the symbiotic association (Horn *et al*., 2001; Horn *et al*., 2000). The knowledge about bacterial endosymbionts of *Acanthamoeba* is still in its infancy. The association of BEs and the T types of their host *Acanthamoeba* is a vital area which needs to be investigated in detail to establish their preference for a particular T type, if any.

1.1.9.2.4. *Acanthamoeba* as training grounds

The survival of bacterial pathogens in *Acanthamoeba* is of greater significance because of the remarkable similarity with macrophages. Both are unicellular, use amoeboid movement, employ phagocytosis to capture food and bacteria, share cellular structures, features and physiological processes. *Acanthmoeba* and macrophages are, therefore, evolutionarily considered to be related (Siddiqui and Khan, 2012). It is believed that the bacterial mechanisms for survival developed and evolved in *Acanthamoeba* were later used to survive in the macrophages. This leads to the hypothesis that *Acanthamoeba* served as training grounds for bacteria in their adaptation as animal pathogens (Abd *et al*., 2009; Cirillo *et al*., 1997; Siddiqui and Khan, 2012; Tenant and Bermudez, 2006).

The most thoroughly studied example of such interaction is that of *L. pneumophila* and *Acanthamoeba. L. pneumophila* are believed to have evolved and adapted as parasites of *Acanthamoeba*. Such interaction served as a preliminary process in the adaptation of these bacteria from primitive form of macrophages (*Acanthamoeba*) to macrophages of multicellular animals (Abu Kwaik *et al*., 1998). It was found that *L. pneumophila* which normally parasitize human macrophages, could survive in *Acanthamoeba* as well (Rowbotham, 1980). *L. pneumophila* use remarkably similar strategies for internalization and survival/proliferation in *Acanthamoeba* as used for macrophages (Table 1.3).

Table 1.3: Comparison of various mechanisms involved in the interaction of *L. pneumophila* with macrophages and FLA showing greater similarity in both, which suggests the survival in amoebae might be pre-adaptive in successful survival of these bacteria in human macrophages.

Mechanism	**Macrophages**	**Amoebae**
Entry by coiling phagocytosis	Yes[1]	Yes[4]
Phagosome-lysosome fusion	No[1]	No[4]
Phagosome association with RER	Yes[2]	Yes[5]
Replication in phagosome	Yes[3]	Yes[6]
Host cell lysis	Yes[3]	Yes[6]
Involvement of *icmT, icmR, icmQ, icmP, icmO, icmM, icmL, icmK, icmE, icmC, icmD, icmJ* and *icmB* genes in intracellular survival	Yes[7]	Yes[7]
Requirement of Dot/Icm type IV secretion system for intracellular proliferation by evading endocytic pathway	Yes[7,8]	Yes[7,8]
Requirement of heavy metal efflux gene island in *L. pneumophila* for intracellular survival or replication	No[9]	No[9]

[1](Bozue and Johnson, 1996) ; [2](Abu Kwaik, 1996) ; [3](Rowbotham, 1980) ; [4](Horwitz, 1983); [5](Swanson and Isberg, 1995) ; [6](Horwitz and Silverstein, 1980); [7](Segal and Shuman, 1999); 8(Al-Khodor *et al*., 2008); (Kim *et al*., 2009).

Therefore, *Acanthamoeba* seem to have remarkable characteristics to act as training sites for the evolution of bacteria as pathogens.

1.1.9.2.5. Survival of bacteria in cysts

A number of bacteria have been identified that can successfully survive within the *Acanthamoeba* cysts including *L. pneumophila* (Kilvington and Price, 1990), *Vibrio cholerae* (Thom *et al*., 1992), *Francisella tularensis* (Abd *et al*., 2003; El-Etr *et al*., 2009), *Mycobacteria* (Adekambi *et al*., 2004; Thomas and McDonnell, 2007) and *Vibrio mimicus* (Abd *et al*., 2010). Cysts also provide protection to the internalized bacteria against environmental hazards. For example, the experiments conducted by Kilvington and Price (1990) indicated successful survival of ingested *L. pneumophila* within cysts and protection against high levels of free chlorine (50 mg/L) for the length of experiment (upto two weeks). Abd *et al*. (2003) found *Francisella tularensis* witin the cysts of *A. castellanii* even after 40 days of co-culture. The ability of bacteria, especially human pathogens, to survive within the *Acanthamoeba* cysts is alarming as the highly robust cysts not only protect the residing bacteria from the extreme harmful environmental and chemical effects but they also serve as carriers for them (Thomas *et al*., 2010).

1.1.10. Interaction of *Acanthamoeba* with emerging human pathogens-*Arcobacter butzleri* and *Rhodococcus equi*

The detailed interaction of *Acanthamoeba* with two relatively distinct bacterial pathogens, *Rhodococcus equi* (Gram +ve and aerobic) and *Arcobacter butzleri* (Gram –ve and microaerophilic), was studied in this thesis both of which are emerging human bacterial pathogens (Calvo *et al*., 2013; Weinstock and Brown, 2002). Their interaction with *Acanthamoeba* and, therefore, the impact of this relation has not been thoroughly studied previously. The following description gives an account of these pathogens.

1.1.10.1. *Arcobacter butzleri*

Arcobacter is classified within the family *Campylobactereaceae* together with the genera *Campylobacter* and *Sulfurospirillum* (Ferreira *et al*., 2015) and comprises of 18 species isolated not only from environment but also from animals and humans (Sasi Jyothsna *et al*., 2013). *Arcobacter* is phenotypically similar to *Campylobacter* except its ability for aerotolerance and growth at lower temperatures (On *et al*., 1996; Vandamme *et al*., 1992). *Arcobacter* has received great attention in recent years because of its potential as an emerging food borne pathogen (Cardoen *et al*., 2009; Collado and Figueras, 2011). *Arcobacter* species have been found from various human food supplies. Very recently 25% of the seafood sold in retail markets of Mumbai in India have been found to be contaminated with *Arcobacter* sp. (Rathlavath *et al*., 2015). In another study the prevalence of *Arcobacter* sp. in broilers was found to be 43.0% while 18.2% in turkey (Atanassova *et al*., 2008).

A. butzleri is the species most commonly associated with human infections in the form of enteritis causing diarrhoea (Prouzet-Mauleon *et al*., 2006; Vandamme *et al*., 1992; Vandenberg *et al*., 2004). It has also been found without signs in GI tract of farm animals (Hume *et al*., 2001). Once the bacteria are excreted by their host, they disseminate in the environment and have the potential to survive there and disseminate (Collado *et al*., 2008). *A. butzleri* has been found in various types of water including drinking, well, canal, sewage and river water (Diergaardt *et al*., 2004; Jacob *et al*., 1993; Rice *et al*., 1999; Stampi *et al*., 1999).

A. butzleri are Gram-negative. They are motile, non-spore-forming and curved to S-shaped rods. The dimensions are 0.2-0.9×0.5-3 μm and the bacteria are capable of growing aerobically/anaerobically from 15-42°C while microaerophilic conditions are required for optimum growth without hydrogen (Vandamme, 2000). Infection is believed to be contracted by ingestion of contaminated food or water, however, the exact mechanisms involved in the pathogenicity and transmission of this pathogen are unclear (Levican *et al*., 2013).

The interaction of *Campylobacter* which are closely related to *Arcobacter* has been studied in detail. *Acanthamoeba* has been shown to act as a potential reservoir for *C. jejuni*. The bacteria can infect *Acanthamoeba* cells *in vitro* and the internalized bacteria localized in vacuoles have greater survival. The role of *Acanthamoeba* as potential reservoir for *C. jejuni* in the environment is a greater concern regarding the epidemiology of this pathogen as it has a broad host range (Axelsson-Olsson *et al*., 2005).

Because of the very robust nature of *Acanthamoeba* being capable of interacting with a very wide variety of organisms, it is probably not possible to completely understand the dynamics of interaction and infection of *Acanthamoeba* by studying a particular pathogen. Therefore, the more the knowledge of the ways *Acanthamoeba* interact with different pathogens the better the understanding of this versatile organism will emerge with a broader picture that will unveil important aspects of the host-pathogen relation (Siddiqui and Khan, 2012). It is hoped that the current study will add to the knowledge of *Acanthamoeba*-bacteria interaction.

1.1.10.2. *Rhodococcus equi*

Rhodococcus equi, the Gram+ve actinomycete, are closely related to mycobacteria. They are versatile organisms and are present in soil as well as faeces of herbivores (Barton and Hughes, 1984). They are primarily important equine bacterial pathogens and the causative agents of severe respiratory distress in foals (1-6 months) resulting in significant economic losses to equine breeding industry. They cause subacute or chronic bronchopneumonia and can spread to other parts of the body resulting in

mesenteric lymphadenitis, osteomyelitis, purulent arthritis, reactive arthritis, and ulcerative lymphangitis. Infection can get worse and even result in death in untreated cases (Meijer and Prescott, 2004; Prescott, 1991). They can also infect a variety of other animals (Horowitz *et al*., 2001; Prescott, 1991) and even immunocompromised humans (Prescott, 1991). They can replicate in the intestine and thus faeces serve as a potential source for dissemination of virulent bacteria from foals. They can survive the hostile environment of macrophages and the virulence is related to the possession of the virulence plasmid (Takai *et al*., 2000b; Takai *et al*., 1999).

R. equi are non-flagellate (Prescott, 1991), but some strains can have appendages or pili (Nordmann *et al*., 1994). Optimal temperature for growth is 30-37°C (Prescott, 1991) although the reported range is 10-40°C (Walsh *et al*., 1993). *R. equi* can not only use carbon from organic acids like proprionate or acetate (abundantly present in manure of herbivores) (Prescott, 1987; Prescott, 1991) but they can also utilize lipids (in macrophages) in a similar manner to mycobacteria, but not sugars. They are surrounded by thick polysaccharide capsule (Prescott, 1991) and can survive some extreme environmental conditions like oxidative stress and low pH (Benoit *et al*., 2002; Benoit *et al*., 2000).

R. equi cause infection in foals up to four months of age (Zink *et al*., 1986) resulting in fever and respiratory distress usually with pus filled lung abscesses (Lavoie *et al*., 1994) if left untreated this condition may cause asphyxiation and death. Dissemination of infection can lead to diarrhoea and vertebral osteomyelitis (Prescott, 1994). Ingestion or introduction of the organism through breached skin can cause intestinal or wound site ulcers respectively (Bell *et al*., 1998).

Immunosuppressive conditions like AIDS put humans at greater risks of *R. equi* infection resulting in high mortality. The lungs are the most commonly affected organs (Bell *et al*., 1998; Hsueh *et al*., 1998). *R. equi* can also cause disease (usually respiratory infections) in a wide range of other animals including pigs, cattle, goats, sheep, llama, cats and dogs (Muscatello *et al*., 2007; Prescott, 1991).

The virulence plasmid of *R. equi* bears a pathogenicity island (PI) containing, among other genes, the virulence associated protein (*vap*) family consisting of *vapA* among seven genes (Polidori and Haas, 2006; Takai *et al*., 2000a) together with two pseudo-*vap* genes (Russell *et al*., 2004). The *vapA* encodes VapA which is a virulence factor and is required for survival and growth in macrophages (Giguere *et al*., 1999; Jain *et al*., 2003). Several copies of *vapA* may be present but can be lost at 38°C (Chirino-Trejo and JF, 1987; Takai *et al*., 1991).

The interaction of *R. equi* with *Acanthamoeba* has not been studied, however, this has been investigated for the closely related *Mycobacterium*. In an investigation of interaction of environmental mycobacteria, using 26 species of *Mycobacterium*, it was shown that all the species had potential to survive in *Acanthamoeba* for five days while they could survive in cysts for up to 15 days (Adekambi *et al*., 2006). *Mycobacterium kansasii* was also found to be able to grow inside *Acanthamoeba* (Goy *et al*., 2007). In another study *M. smegmatis* demonstrated its ability to survive and grow in *Acanthamoeba* while the co-culture resulted in increased growth of bacteria (Lamrabet *et al*., 2012).

Investigation of intracellular survival of *R. equi* in *Acanthamoeba* is significant not only to establish the current potential of *Acanthamoeba* in acting as an environmental reservoir of *R. equi* but also it is important in establishing the evolutionary role of protists, like *Acanthamoeba*, as the "missing link" in the transformation of these bacteria from saprophytic to intracellular pathogens. The PI of *R. equi* is of prime importance in determining virulence as it harbours virulence genes and is believed to have been acquired through horizontal gene transfer (HGT) (Letek *et al*., 2008). The journey starting from the acquisition of virulence genes by non-pathogenic saprophytic *R. equi* (through HGT) until their transformation as pathogens definitely required a missing link in the environment which served as an evolutionary pre-adaptive platform in evolving potential to withstand hostile cellular environment of macrophages and thus cause human and animal infections. *Acanthamoeba*, being FLA in the environment and having close resemblance with macrophages, are potentially strong candidates for this missing link which is explored in this study as well (Chapter 4).

1.1.11. Aims and objectives of the study

This study aims at investigating the impact of *Acanthamoeba*-bacteria interaction on two named organisms as well as its possible implications on human health. Therefore, the objectives of this study can be broadly divided into three categories i.e. 1) effect of bacteria on *Acanthamoeba*, 2) effect of *Acanthamoeba* on bacteria, and 3) assessment of possible consequences of *Acanthamoeba*-bacteria interactions on human health.

E. coli is usually used as the monoxenic source of food for isolation of *Acanthamoeba* and in this way 19 T types of *Acanthamoeba* have been recognized worldwide. This either means that *E. coli* is the perfect source and every genotype of *Acanthamoeba* can be successfully isolated, or it implies that despite being a good food, all the genotypes cannot be recovered equally well so some genotypes can go masked and this can ultimately affect the outcomes of the prevalence or diagnostic studies. So the first aim was to investigate the role of bacteria on determining the diversity of *Acanthamoeba*. This was done by using *E. coli* in parallel with other bacteria (in this study the Gram+ve *Enterococcus* and Gram-ve *Arcobacter*) (Figure 1.11-a) for the isolation of *Acanthamoeba* from environmental samples (soil) separately and the *Acanthamoeba* isolates so obtained were compared to decide whether the use of a single bacterial type can affect the outcomes of a prevalence study (Figure 1.11-b) and ultimately the control and treatment strategies (Figure 1.11-c).

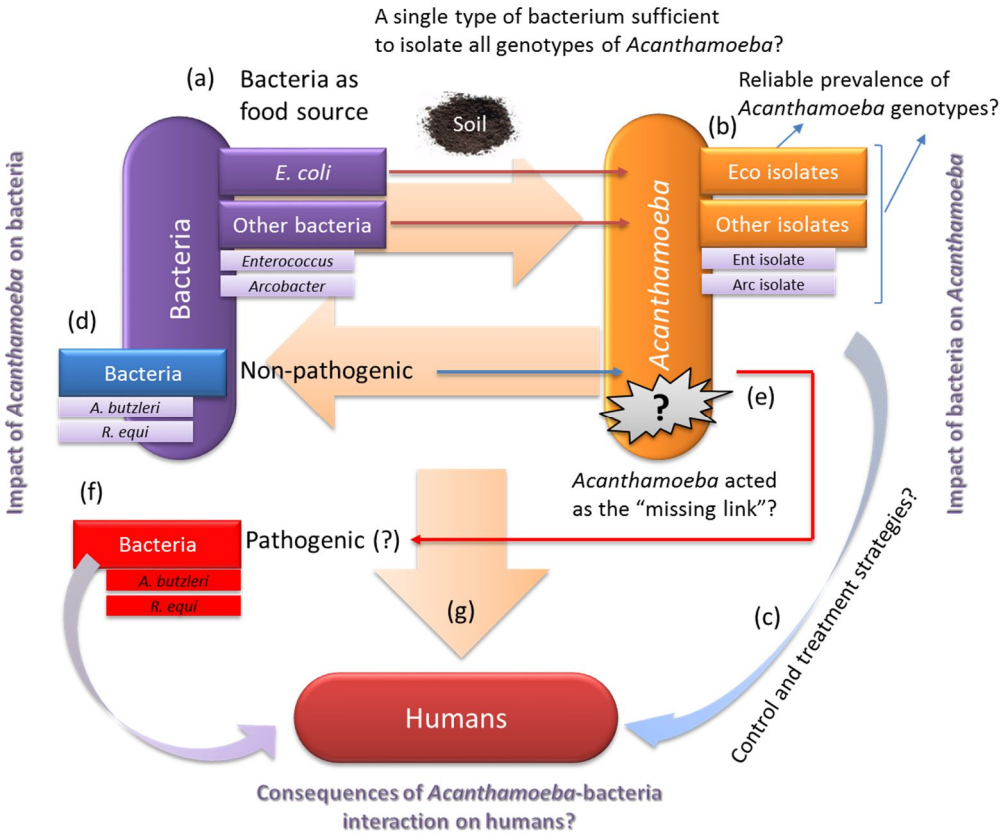


Figure 1.11: An overview of objectives of the current research that involved study of *Acanthamoeba*-bacteria interaction to understand influence of both the organisms on each other and the consequences of this interaction on human health. First aim involved investigating influence of bacteria (as food) on recovery and diversity of *Acanthamoeba* genotypes addressing question "is *E. coli* alone, used as food source for isolation, capable of recovering all the genotypes of *Acanthamoeba* from a sample (soil). This was studied by using other bacteria in addition to *E. coli* (Gram+ve *Enterococcus* and Gram-ve *Arcobacter*) (a) for isolation of *Acanthamoeba* followed by genotyping and characterization to compare *Acanthamoeba* isolates on different bacteria. Dissimilar results (more genotypes recovered than test bacteria, *E. coli*), implies that using only a specific bacterium (*E. coli*) cannot result in reliable prevalence of *Acanthamoeba* genotypes (b) which can subsequently affect the control and treatment strategies (c). The effect of *Acanthamoeba* on bacteria was assessed by studying intracellular survival of bacteria (emerging human pathogens *A. butzleri* and *R. equi*) (d) that was used to test the role of amoebae in acting as the "missing link" (e) in the evolution of non-pathogenic bacteria into pathogenic (f). Together, the results of interaction between *Acanthamoeba* and bacteria were accessed for direct effect on the human health (g).

The effect of *Acanthamoeba* on bacteria, as reservoirs, was investigated by studying the intracellular survival capabilities of bacteria (emerging human bacterial pathogens *A. butzleri* and *R. equi* in this study) (Figure 1.11-d) to test the possible role of *Acanthamoeba* in acting as reservoirs and training grounds (Figure 1.11-e) which may enhance or select their virulence properties (Figure 1.11-f) enabling them to infect

humans. The possibility of how these *Acanthamoeba*-bacteria interactions can have ultimately an influence on human health was the final objective. The detailed objectives are discussed with each chapter while the main objectives are grouped and described below.

1.1.11.1. Impact of bacteria on *Acanthamoeba*

This has been addressed in Chapter 2 and involved studying the role of a particular type of bacterium in isolating various genotypes of *Acanthamoeba*. However, owing to the variety of *Acanthamoeba* isolation techniques and limitations in the form of contamination and delayed isolation times, the isolation procedure was first optimized. Therefore, the main objectives included:

- To optimize the procedure for isolation of *Acanthamoeba* from soil to reduce chances of contamination and minimizing overall time of isolation.
- To isolate *Acanthamoeba* on different prey bacteria (including *E. coli* as control) and to compare the genotypes recovered to assess any effect of choice of bacterium on recovery of *Acanthamoeba* genotypes based on genotyping, morphological and pathological characteristics and endosymbiotic profiles.

1.1.11.2. Impact of *Acanthamoeba* on bacteria

This has been addressed in Chapter 3 and 4 and involved in studying the role of *Acanthamoeba* on bacteria as environmental reservoirs, focusing on the implications on human health and evolutionary significance of *Acanthamoeba* in serving as pre-adaptive grounds for emerging human pathogens *A. butzleri* and *R. equi*. Therefore, the main objectives included:

1.1.11.2.1. To investigate the survival of *A. butzleri* in *Acanthamoeba* (Chapter 3) focusing on the following points:

- To study how *Acanthamoeba* and *A. butzleri* interact at the cellular and molecular levels including chemotactic location, internalization of bacteria, role of sugars, and intracellular signalling pathways on bacterial uptake.

- To study the pathogenic potential of *A. butzleri* towards *Acanthamoeba*.
- To study the effect of intracellular survival in *Acanthamoeba* on pathogenicity of *A. butzleri*.
- To study the mutual effect of co-culture on both *A. butzleri* and *Acanthamoeba*.
- To study the effect of modulation of gene expression of *A. butzleri* on their pathogenicity towards *Acanthamoeba*.

1.1.11.2.2. To investigate the survival of *R. equi* in *Acanthamoeba* (Chapter 4) focusing on the following points:

- To access the pathogenic potential of *R. equi* towards macrophages focusing on the role of virulence plasmid and temperature on the pathogenicity of *R. equi*.
- To study and compare the role of virulence plasmid and temperature in macrophages on the pathogenicity of *R. equi* towards *Acanthamoeba*.
- To investigate the possible role of *Acanthamoeba* as reservoirs for the selection of pathogenic potential of *R. equi* which may pose serious threat in the form of virulent human infections.

Finally, to deduce, based on the outcomes of the above objectives, the possible consequences of *Acanthamoeba*-bacteria interactions on human health.

Chapter 2

Impact of bacteria on *Acanthamoeba*: Role of bacteria in the diversity of *Acanthamoeba*

Abstract

This chapter focuses on the impact of bacteria on *Acanthamoeba* investigating the influence of prey bacteria on the diversity of *Acanthamoeba* genotypes and probing into the question that "can the presence of a particular type of bacteria play role in the diversity of *Acanthamoeba* by masking and/or favouring certain genotypes of *Acanthamoeba*?" However, owing to the variety of variations in the isolation methods, the procedure for isolation of *Acanthamoeba* was first optimized. A number of parameters were fine tuned for this purpose including enrichment of samples, spreading techniques to minimize contamination, easing transition phase from monoxenic to axenic culture by introduction of bacteria and changes in growth medium. The optimized method resulted in significantly shorter overall time for isolation and adaptation to liquid culture medium as compared to the traditional method. The method was then applied to investigate the above question by using three different types of bacteria (*E. coli*, *Enterococcus* and *Arcobacter*) separately for isolation of *Acanthamoeba* from a total of 102 soil samples randomly collected from various parts of England and Scotland. *Acanthamoeba* were isolated to have *E. coli* (Eco), *Enterococcus* (Ent) and *Arcobacter* (Arc) isolates of *Acanthamoeba*, making a total of 306 sample processings. It was revealed that the presence of different bacterial types could affect the genotypes of *Acanthamoeba* recovered specially at the subgroup and subtype level. The effect was most prominent in case of Arc isolates which showed greater diversity of 18S rRNA sequences than Eco isolates. T types recovered for Eco isolates included T2(6%), T4(89.2%), T11(2.4%) and T13(2.4%); for Ent isolates included T4(95.1%), T16(3.7%) while 1.2% of isolates were intermediate T13/T16; for Arc isolates included T2(14.3%), T2/6(2.4%), T4(78.6%), T13(2.4%) while 2.4% sequences were intermediate T13/T16. There were also remarkable differences among the T4 types on the basis of subgrouping. Eco isolates had T4-A (54.1%), T4-B (16.2%), T4-C (1.3%), T4-D (8.1%), T4-E (9.5%), T4-N (10.8%); Ent isolates had T4-A (47.0%), T4-B (7.4%), T4-C (11.1%), T4-D (11.1%), T4-E (12.3%), T4-N (11.1%); and Arc isolates had only T4-A (28.8%), T4-B (19.7%), T4-E (34.8%) and T4-N (16.7%). Although pathogenic potential (thermotolerance and osmotolerance) was not very helpful, morphology of cysts as well as the trends for harbouring bacterial endosymbionts among the three isolates was quite varied. The Arc isolates had 15.7% bacterial endosymbionts as compared to 7.8% of Eco and 12.9% of Ent isolates, while T4-B was found to be most susceptible for endosymbionts. Together these results indicate a definite role of bacteria on the diversity of *Acanthamoeba* genotypes and suggest consideration for using different types of bacteria for isolation of *Acanthamoeba* to help surface the masked populations as well.

2.1. Introduction

E. coli are in general used for the monoxenic isolation of *Acanthamoeba* from soil and other samples (Gatti *et al*., 2010; Landell *et al*., 2013; Liang *et al*., 2010; Rahdar *et al*., 2012; Reyes-Batlle *et al*., 2014; Rivas *et al*., 2004; Tanveer *et al*., 2013; Todd *et al*., 2014). Following their use by different workers across the world, 19 T types of *Acanthamoeba* have been described (Corsaro and Venditti, 2010; Gast *et al*., 1996; Hewett *et al*., 2003; Horn *et al*., 1999; Lanocha *et al*., 2009; Magnet *et al*., 2014; Nuprasert *et al*., 2010; Qvarnstrom *et al*., 2013; Stothard *et al*., 1998) while T20 has also been suggested (Fuerst *et al*., 2014). These have been discussed later in this chapter along with *Acanthamoeba* subgroups (T4-A, -B, -C, -D, -E, -F & -N and T2/6-A, -B, & -C) and subtypes (T4-1 to T4-38). T4 has consistently been described as the major T type recovered (Fuerst, 2014; Maciver *et al*., 2013). Whether we know of these types of *Acanthamoeba* because *E. coli* have been used for the isolation, which is a cause of concern, or do there exist other types or subtypes of *Acanthamoeba* in the environment that might have been isolated if bacteria other than *E. coli* were used? This vital issue has never been elaborated in detail which is the main objective of this chapter. It required the use of *E. coli* (as control) and other types of bacteria as food source for the isolation of *Acanthamoeba* from samples (soil). However, because of the variations and the long isolation times it was important to optimize the *Acanthamoeba* isolation procedure. Therefore, the first phase of the study deals with optimization of procedure for isolation of *Acanthamoeba* from soil.

2.1.1. Isolation of *Acanthamoeba* from soil

The various methods for isolation of *Acanthamoeba* from soil, in general, have similar steps although some variations do exist. The typical traditional method for isolation of *Acanthamoeba* from soil (Maciver *et al*., 2013) is shown in Figure 2.1 and consist of two main phases i.e. recovery phase and axenization phase. During the recovery phase *Acanthamoeba* are isolated and purified by repeated passages on bacteria overlaid on the surface of non-nutrient agar. However, they are not adapted to grow in the absence of bacteria (axenically) and, therefore, need time for adaption to axenic mode of life in growth media only. The method of isolation of *Acanthamoeba* is a laborious and

time consuming procedure that usually takes weeks or months (Zanella *et al*., 2012). Therefore, there is requirement for simple modifications that may reduce the overall time for isolation and adaptation of *Acanthamoeba*.

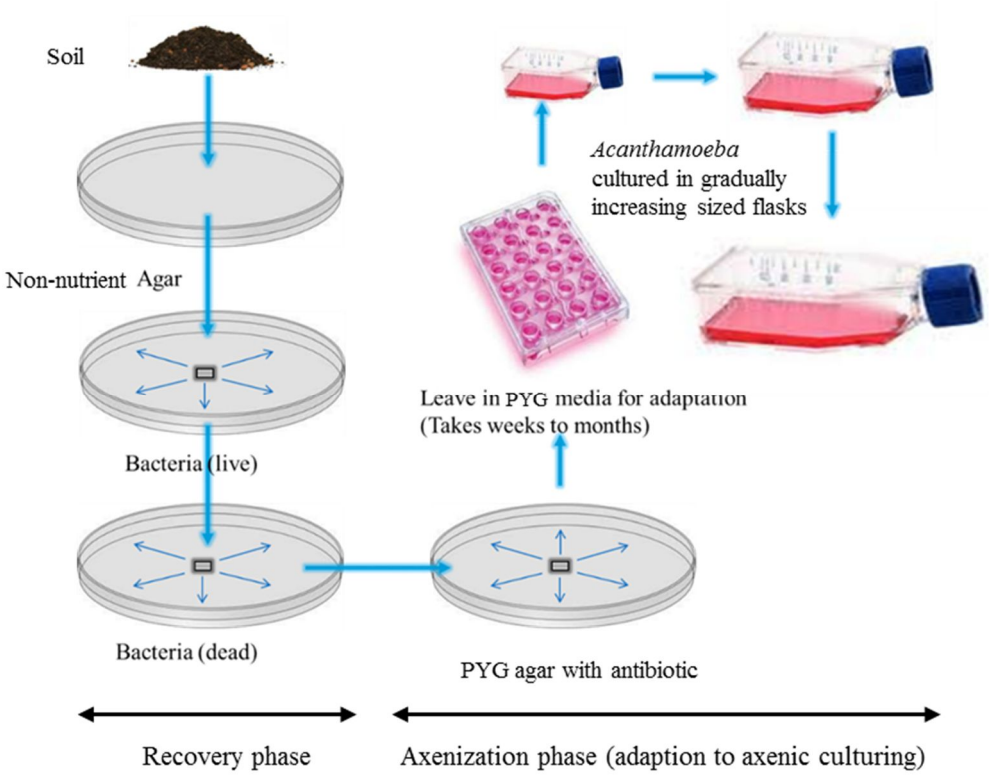


Figure 2.1: A diagrammatic representation of the usual method for the isolation of *Acanthamoeba* from soil although some variations exist. The soil sample is spread onto the surface of a non-nutrient agar plate from which a small cube of agar is cut out and held face down onto the surface of another agar plate with live bacteria overlaid as source of food. The process of transfer is repeated a few more times with dead bacterial agar plates before transferring to nutrient agar plate in the presence of antibiotics but no bacteria. Adaptation to liquid culture media is exercised by transferring amoebae from solid media to liquid culture which is then scaled up as amoebae start to adapt and grow.

Although the isolation of *Acanthamoeba* is in generally made using the classical method there are some modifications although the basic philosophy remains the same i.e. selective growth of *Acanthamoeba* on bacteria (*E. coli*) with subsequent steps for further purification and axenization. Similar method has been used by Rezaeian *et al*., (2008) and Rahdar *et al*., (2012) that involved dissolving the soil samples (100-200 g) in water that were then processed as for water samples i.e. filtering the water through 0.45 µm filter papers and these were then inverted onto 1.5% non-nutrient agar

medium followed by observation for two months. The cysts from the less contaminated areas were collected and transferred to new agar plates.

Reyes-Batlle *et al*., (2014) dissolved soil samples in 20 mL Neff's saline and used a 150 µL aliquot to be transferred onto the surface of the non-nutrient agar plates that were then incubated at 25ºC for seven days. Any positive samples were subsequently diluted and transferred to new plates. The isolated *Acanthamoeba* were then transferred to PYG media for axenization. Similar procedure for isolation of *Acanthamoeba* was used by (Lorenzo-Morales *et al*., 2005) and the initial observation for appearance of *Acanthamoeba* was made for up to two weeks, however, no mention of the period required for axenization is mentioned. Kong *et al*., (1995) used 1 g of soil that was spread onto the surface of non-nutrient agar plate overlaid with dead *E. coli*. The plates were observed for growth for one week. A block of agar with amoebae was cut out and treated with 0.1N HCl before transferring to the PYG media. The same method was used by Chung *et al*., (1996). Ertabaklar *et al*., (2007) also used 1 g sample of soil for spreading onto *E. coli* agar plates. Tsvetkova *et al*., (2004) used for axenization of *Acanthamoeba* two liquid media i.e. PPG which is proteose-peptone glucose (1.5%, 1.8% in Page's amoeba saline) and yeast extract-PAS medium (YAS) which is 0.1 g yeast extract (Merck) in 1,000 ml Page's amoeba saline).

Whatever the modification is used, the isolation and axenization of *Acanthamoeba* is a time consuming task. The challenges of overcoming contamination and the slow axenization process make the method more labour intensive. A variety of fungal contamination from environmental samples like soil is quite expected (Figure 2.2) and in some cases it may be difficult to avoid fungal contamination even after rigorous treatment like HCl, thus putting the precious sample at risk (Corsaro and Venditti, 2010) which may have to be discarded because of excessive contamination (Kilic *et al*., 2004). This can particularly be a problem when the number of samples is large. This necessitates additional modifications to be introduced which are simple and practical to be followed for efficient recovery of amoebae.

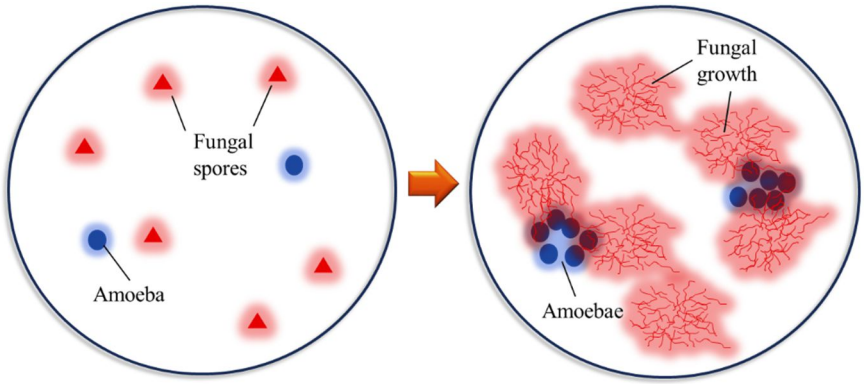


Figure 2.2: Spread of fungal contamination that is usually encountered during isolation of *Acanthamoeba* from soil samples. This is specially a problem if the number of amoebae in sample is lesser than surrounding fungal contamination which results in amoebae trapped within a fungal mass. This kind of samples either require rigorous treatment for recovery of amoebae or these have to be discarded.

A modification of isolation was reported by Zanella *et al*., (2012) who picked up individual cysts with the help of micromanipulator needle and transferred them to the new plate, although the use of micromanipulator for the cloning purpose of *Acanthamoeba* isolates had already been documented by others (Ertabaklar *et al*., 2007; Tsvetkova *et al*., 2004; Walochnik *et al*., 2000a). However, this method is not only time consuming but also require special equipment and skills to carry out the isolation of individual cysts. Moreover, only ten samples were processed which may not reflect diverse strains of *Acanthamoeba* found in various environmental conditions.

2.1.1.1. Avoiding contamination

There is always a need for simple modifications in the isolation process to avoid problems like contamination especially when dealing with large number of samples to speed up the process. One such possibility is explained in Figure 2.3 which has been explored in this chapter. It aims at enrichment of soil samples (before processing to increase the number of amoebae in sample by adding bacteria) and dilution/spreading technique (dilution of enriched samples to decrease the density of fungal contamination as well). When spread onto agar plates with bacteria, the chances that at least one of the plates will have pure or at least less contaminated culture of amoebae which can easily be separated and purified, are increased.

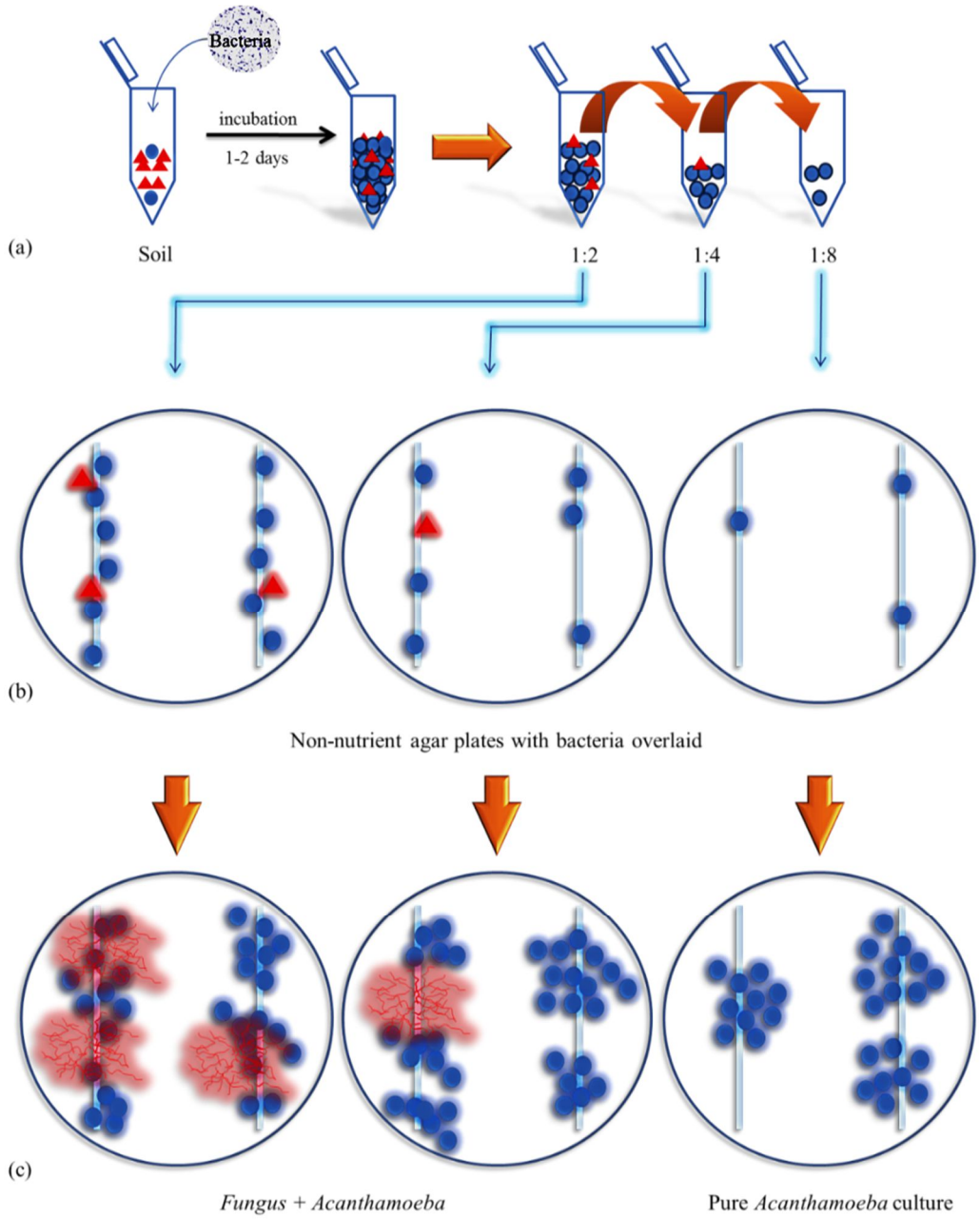


Figure 2.3: Diagrammatic representation of the strategy used in modified method for minimizing contamination during isolation of *Acanthamoeba* from soil showing the simple concept of how the selective growth of amoebae may be encouraged leaving fungal growth minimized. (a) Soil sample and washed live bacteria (which serve as the main source of food) were mixed together in a small tube. *Acanthamoeba* (circles) could comparatively show better growth by consuming bacteria while there were less chances for finding enough food for fungi (triangles). This was further improved by diluting the above soil suspension two-fold in saline. There were chances that one or more of these dilutions had no or very low contamination. (b) This could be visualized by taking a small aliquot from each of the diluted suspension and spreading it edge-to-edge on the surface of an agar plate overlaid with live bacteria. (c) By incubating the plates and allowing the amoebae to grow the plates with no or minimum contamination could be identified.

2.1.1.2. Easing the transition phase

Attempts can be made during transition period, from bacteria-eating monoxenic phase to axenic phase when only culture media is given as food source, to help amoebae adapt to new culture conditions smoothly. In general, amoebae are directly transferred from non-nutrient agar plates with dead bacteria to nutrient agar plates or nutrient media (Bagheri *et al*., 2010; Gatti *et al*., 2010; Rahdar *et al*., 2012; Reyes-Batlle *et al*., 2014) as shown in Figure 2.1. This abrupt change can stress the amoebae and delay the adaptation time. Amoebae are used to phagocytosing bacteria as a food source in the environment, therefore, the addition of bacteria on the nutrient agar plate is a logical modification that can ease the stress. This can further be assisted by the addition of dead bacteria even in the culture media (Figure 2.4).

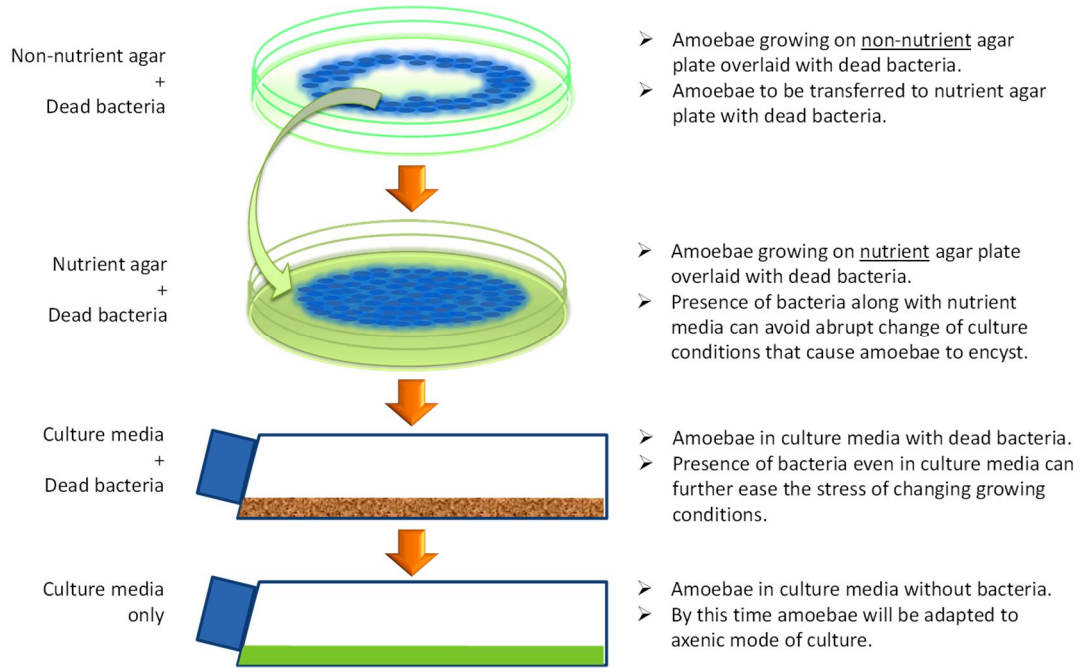


Figure 2.4: Attempt to reduce the stress of abrupt change of culture conditions during isolation of amoebae from bacteria-eating monoxenic phase to culture-media-only phase. Unlike traditional method (Figure 2.1) instead of transferring amoebae from non-nutrient agar plate with dead bacteria to nutrient agar, addition of dead bacteria to nutrient agar can ease the effect of sudden change. This can further be assisted by addition of dead bacteria even in liquid culture media. This can overall help amoebae adapt to axenic mode of culture quickly.

2.1.1.3. Choice of culture media for *Acanthamoeba*

A large number of *Acanthamoeba* strains/species have been isolated from clinical samples. Many of these could be grown on the basic PYG medium used for the free-living isolates (Kong *et al*., 1995; Lorenzo-Morales *et al*., 2005; Reyes-Batlle *et al*., 2014; Schuster, 2002; Visvesvara *et al*., 1975). The PYG contains peptone, yeast extract and glucose with varying proportions of these components. The original medium used by Neff consisted of these ingredients at 0.75%, 0.75% and 1.5% respectively (Neff, 1957). Other media have also been tried. A carbon source has been found to be an important component to avoid encystation (Byers *et al*., 1980). Glucose was found to have positive effect on growth while acetate had little effect (Byers *et al*., 1980). Inability of acetate to substitute for glucose was also reported by (Ingalls and Brent, 1983).

Different media have been used by different workers with variable results, such as PPG (proteose-peptone glucose) and YAS (yeast extract-Page's amoeba saline) medium (Tsvetkova *et al*., 2004); TYI-S-33 medium consisting of potassium phosphate, potassium phosphate, sodium chloride, casein digest peptone, yeast extract, glucose, L-cysteine, ascorbic acid, and ferric ammonium citrate (Rezaeian *et al*., 2008; Rahdar *et al*., 2012); PYG with added L-cystein (Kong *et al*., 1995). Different species-specific defined media for *Acanthamoeba* have also been used. PYG with 18 additional amino acids, vitamin B12 and acetate was used for Neff strain (Adam, 1959). A further modification of this medium with incorporation of biotin was introduced by Band (1961). A medium for *Acanthamoeba rhysodes* consisted of glucose/sodium acetate, seven amino acids, biotin, vitamin B12 and thiamine (Band, 1962). Another medium for different strains of *Acanthamoeba* consisted of methionine, arginine, isoleucine, leucine and valine in basal medium (Adam and Blewett, 1967). However, different growth rates were observed with these media and delayed generation times was another problem. Some media gave good results but were too complicated for routine use, like DGM-21A and DGM-21B (Byers *et al*., 1980). Still some media did not work equally well for all isolates (De Jonckheere, 1980). A suitable culture medium for the set of isolates being worked with is, therefore, essential.

2.1.2. Assessment of the role of prey bacteria in the recovery of *Acanthamoeba* genotypes from the environmental samples

It is interesting to note that the isolation of *Acanthamoeba* from environmental samples like soil is in general made by selective growth of *Acanthamoeba* on bacterial agar plates while *E. coli* are used almost always (Astorga *et al*., 2011; Corsaro and Venditti, 2010; Di Cave *et al*., 2009; Edagawa *et al*., 2009; Ertabaklar *et al*., 2007; Horn *et al*., 1999; Huang and Hsu, 2010; Łanocha *et al*., 2009; Liang *et al*., 2010; Liu *et al*., 2006; Magnet *et al*., 2012; Qvarnstrom *et al*., 2013; Walochnik *et al*., 2000a; Yera *et al*., 2007) although in few cases *Enterobacter aerogenes* (*Klebsiella aerogenes*) have been used (Booton *et al*., 2009; Kilic *et al*., 2004; Tsvetkova *et al*., 2004). However, in natural conditions a number of bacteria exist in the environment with wide variations in their distribution from one place to another and, therefore, there are possibilities for any specific association between a T type and the surrounding bacterial population. This may limit the distribution of *Acanthamoeba* to certain areas. Therefore, an important question is that whether the distribution of *Acanthamoeba* T types be limited by the bacterial population. However, this important relation between the *Acanthamoeba* and the bacteria has never been studied in details by linking the bacterial type and the T types of *Acanthamoeba* which is the main objective of this chapter.

Assessment of the role of prey bacteria in the recovery of *Acanthamoeba* genotypes from the environmental samples can be performed by: 1) Isolation of *Acanthamoeba* from the environmental samples (soil) by using *E. coli* in parallel with some other type of bacteria. 2) Characterization of isolated *Acanthamoeba* by genotyping and pathogenic potential. Three different types of bacteria were used in the current study for this purpose, including *E. coli* (as control) in addition to *Enterococcus* and *Arcobacter*. These bacteria were used as these are found in the environment as well and so hoped to better mimic the amoeba-bacteria interaction in the environment under the experimental conditions. Furthermore they cover the Gram-positive/negative spectrum of bacteria.

E. coli are the most extensively studied prokaryotic model organisms for more than six decade. These are Gram-ve rod-shaped facultative anaerobic bacteria which are

commonly found in the intestinal tract of animals. Most strains exist as harmless normal flora of the gut and produce vitamin K2 but some may cause food poisoning. *E. coli* are disseminated in the environment and are also found from various environmental sources. Together with other facultative anaerobes, *E. coli* make up 0.1% of gut flora. *E. coli* can be easily cultured in the laboratory and under favourable conditions they can reproduce in only 20 minutes (van Elsas *et al*., 2011).

Enterococcus are Gram-positive cocci facultative anaerobic organisms. They are usually found in pairs or short chains and were previously considered part of the genus *Streptococcus*. *E. faecalis* and *E. faecium* are most frequently found common commensal organisms in human intestines. They also act as opportunistic pathogens and cause human and animal infections, enterococci are well resistant to a wide range of environmental conditions including temperature (10-45°C) and pH (4.5-10.0). They are also found in a variety of environmental sources including soil, various water sources, beaches and vegetation (Byappanahalli *et al*., 2012).

Arcobacter have already been discussed in detail in Chapter 1 (1.1.3.2.).

2.1.3. Genotyping of *Acanthamoeba*

Genotyping is the most widely accepted subgenus classifications scheme for *Acanthamoeba*. The genotyping of *Acanthamoeba* is based on sequence of 18S rRNA which is ~2300 bp long. The genotypes of T types are differentiated on the basis of full-length 18S rRNA sequences. The suggested standard for differentiation of genotypes is 5% sequence dissimilarity. This means that 18S rRNA sequences of every two different genotypes differ from each other by 5%. So far 20 genotypes (T types) of *Acanthamoeba* have been reported around the world. These are designated as T1 to T20. Full-length sequences have been used for the identification of new genotypes except in case of T15, where GTSA.B1 (genotype-specific amplimer B1) fragment was used (Hewett *et al*., 2003a), and for T16_A, where the Ami fragment was used (Łanocha *et al*., 2009). An overview of the T types (T1-T20) has been shown in Figure 2.5. It is interesting that the T types T13 (Hewett *et al*., 2003) was wrongly reported as T14 (Horn *et al*., 1999). Similarly T20 (Fuerst *et al*., 2015) was wrongly assigned as T4 (Visvesvara *et al*., 2007). The process of improvements in the genotyping system

of *Acanthamoeba* is still an ongoing process and some more modifications in this system have been suggested and are discussed later in this chapter (section 2.1.3.1.).

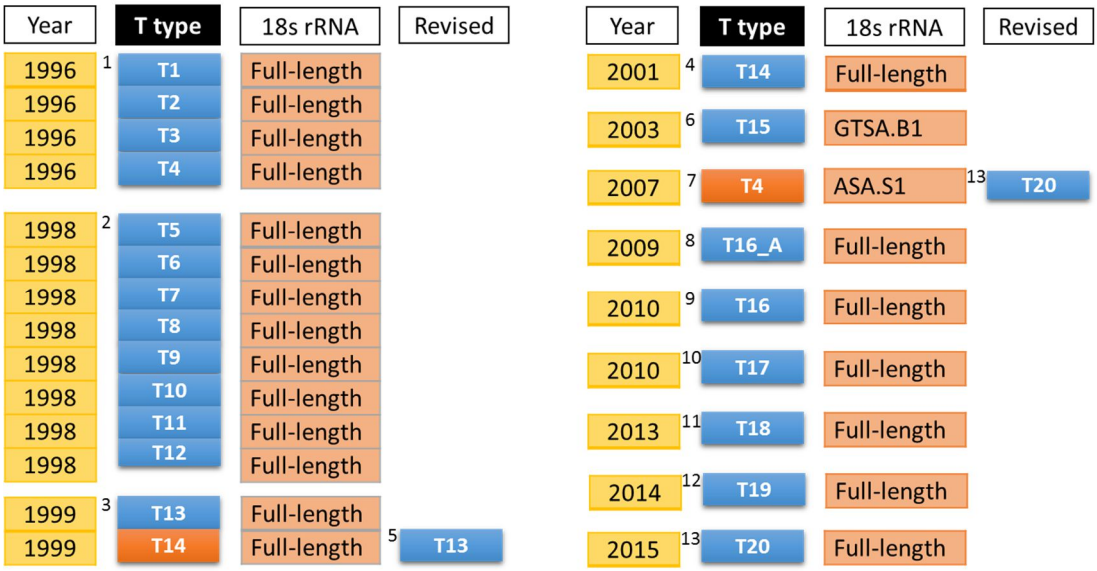


Figure 2.5: An overview of the genotypes (T types) of *Acanthamoeba* based on 18S rRNA sequence showing the timeline of reporting of the genotypes T1 to T20 and the length of 18S rRNA sequence used to claim new genotype. Genotypes T13 and T20 were originally wrongly identified as T14 and T4 respectively. A full-length sequence is normally used to claim a new genotype, however, in case of T15, only GTSA.B1 while in case of T16_A, only Ami fragment was used. Numbers along the T types from 1-13 represent the following references: 1= Gast *et al*. (1996); 2= Stothard *et al*. (1998); 3= Horn *et al*. (1999); 4= Gast (2001); 5= Hewett *et al*. (2003); 6= Hewett *et al*. 2003); 7= Visvesvara *et al*. (2007); 8= Lanocha *et al*. (2009); 9= Corsaro and Venditti (2010); 10= Nuprasert *et al*. 2010; 11= Qvarnstrom *et al*. 2013; 12= Magnet *et al*. 2014; 13= Fuerst *et al*. 2015.

Although a full-length 18S rRNA sequence is usually used to claim a new genotype, for routine purposes smaller fragments are used mostly mainly ASA.S1. The ASA.S1 fragment is also highly genus specific. It is bound by primers JDP1 and JDP2 and is widely used variable region for genotyping. Other fragments include GTSA.B1, Ami and DF3 (Figure 2.6). The GTSA.B1 is bound by primer sequences CRN5 and 1137. It has three variable regions designated as DF1, DF2 and DF3. The DF3 is part of ASA.S1 bound by the primer set 892-JDP2 (~220 bp) and has also been used for subtyping of T4.

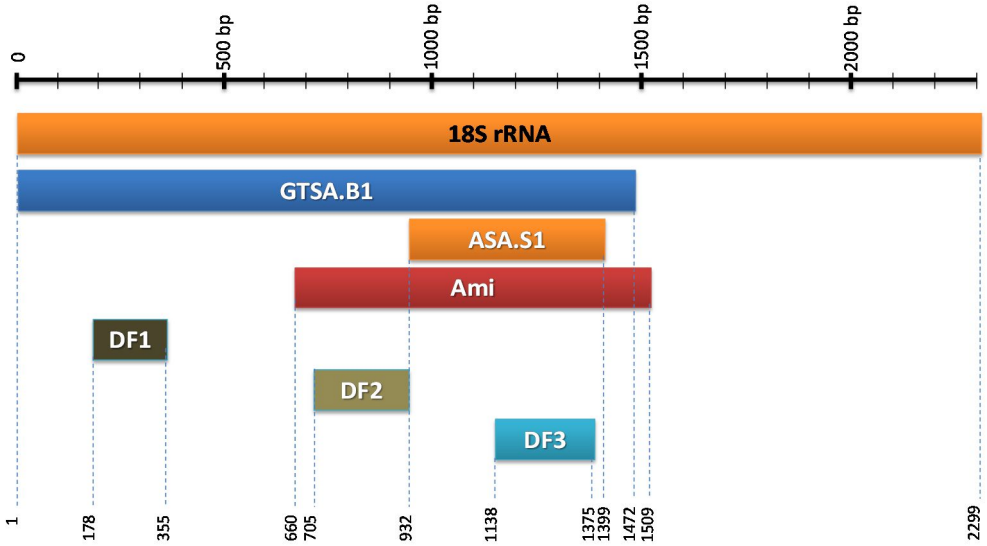


Figure 2.6: Detailed structure of the near full-length sequence of the 18S rRNA of *Acanthamoeba* (T4-U07416.1). **GTSA.B1** fragment (1472 bp) is bound by CRN5-1137 (1-1472 bp), **Ami** fragment (850 bp) by Ami6F1-Ami9R (660-1509 bp) and **ASA.S1** (467) by JDP1-JDP2 (932-1399 bp) while the diagnostic fragments **DF1** (178 bp), **DF2** (228 bp) and **DF3** (238 bp) span the regions from 178-355, 705-932 and 1138-1375 bp, respectively.

Some of the *Acanthamoeba* 18S rRNA sequences are unusually long due to the presence of introns. Among these *A. griffini* (T3) and *A. lenticulata* (T5) have been found to possess introns in their 18S rRNA sequences. Figure 2.7 shows the position of T3 (Genbank Accession No. U07412) and T5 (Genbank Accession No. U94741) introns in 18S rRNA sequences of *Acanthamoeba*. In T3, the intron exists within the GTSA.B1 fragment while in T5 the intron is found outside the GTSA.B1. The presence of introns can affect the sequencing results and these are excluded prior to sequence analysis.

Figure 2.7: *Acanthamoeba* T3 (Genbank Accession No. U07412) and T5 (Genbank Accession No. U94741) genotype with the position of intron being highlighted in grey while the positions of the various primers are located by highlighting in different colours; these include GTSA.B1 fragment, Ami fragment and ASA.S1 fragment.

As discussed in Chapter 1, based on 18S rRNA gene sequence analysis *Acanthamoeba* have been genotypically classified into 19 T types (with proposed T20). Besides this main classification, the whole genotyping system has been reviewed and rearrangements have been made (Fuerst, 2014) in addition to T4 subtyping system (Abe and Kimata, 2010; Booton *et al*., 2002; Ledee *et al*., 2009; Magnet *et al*., 2013; Risler *et al*., 2013; Zhao *et al*., 2010) as explained below.

2.1.3.1. Recent modifications, subgrouping and subtyping of *Acanthamoeba* genotyping system

As more and more sequences are being submitted to international DNA databases, a better understanding of the subgenus classification system of *Acanthamoeba*, based on 18S rRNA, is emerging. Based on the data analysis of submitted sequences, some modifications in the genotypic classification have been suggested recently (Fuerst, 2014; Fuerst *et al*., 2015). This involves segregation of T2/6, as a separate group (containing subgroups T2/6-A, T2/6-B and T2/6-C), and subgrouping of T4 (T4-A, T4-B, T4-C, T4-D, T4-E, T4-F and T4-N). Subgrouping of the T4 was required not only because of the massive abundance of T4 (~1300 T4 sequences out of the ~1800 total genotypes) among the 20 T types but also because of the diverse nature of T4 sequences. It was found upon sequence analysis that the T4 contained well differentiated clades although they didn't meet the 5% sequence diversity rule to be assigned to a new T type but could be subgrouped. The T4 genotypes was, therefore, divided into seven subgroups. The T4 is also divided into various subtypes based on the sequence analysis of the small fragment DF3 (section 2.1.3.1.3.). The overall picture of *Acanthamoeba* genotypic classification that emerges based on 18S rRNA sequence analysis is compiled in Figure 2.8.

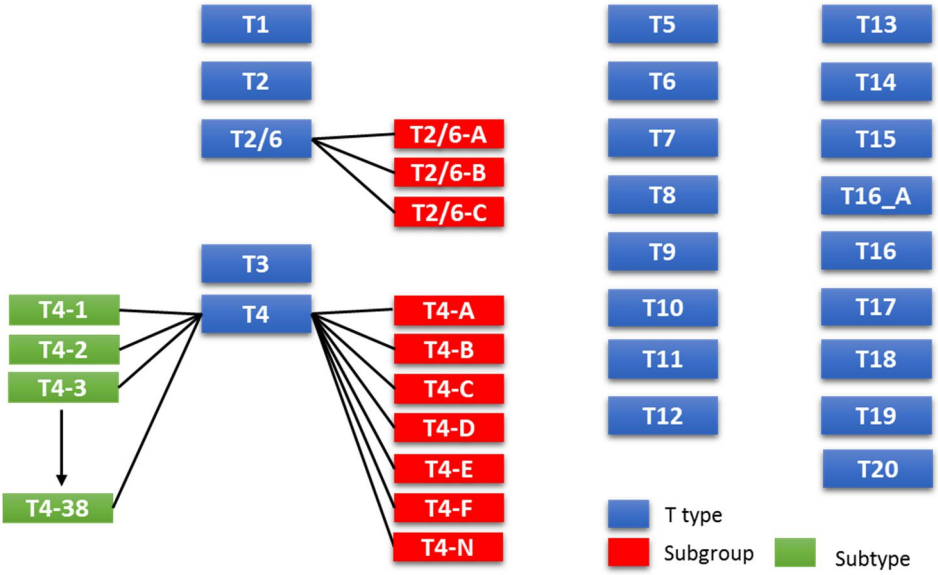


Figure 2.8: The detailed genotyping scheme used for the genotypic classification of *Acanthamoeba* based on 18S rRNA sequence analysis. The T types are mainly categorized from T1 to T19. However, due to intermediary positioning of T types between T2 and T6 these have been placed into T2/6 which are further subgrouped intoT2/6-A, -B and -C. T4 is the main T type and has been further divided into subgroups (T4-A, -B, -C, -D, -E and -F) and subtypes (T4-1 to T4-38). T16_A represents the T16 type reported based on Ami fragment contrary to full-length sequence as in case of T16.

2.1.3.1.1. T2/6

On close phylogenetic examination of T2, T6 and intermediate sequences, the latter were found to be form a separate clade (T2/6) comprised of three subclades (T2/6-A, -B and -C) (Fuerst, 2014; Fuerst *et al*., 2015).

2.1.3.1.2. T4 subgroups

Similarly T4, being the largest T types, has been divided into six subgroups (T4-A, -B, -C, -D, -E and -F) based on the phylogenetic analysis and clades/subclades formation (Fuerst, 2014; Fuerst *et al*., 2015).

2.1.3.1.3. T4 Subtypes

The system for subtyping T4 genotyes was first used by Booton *et al*., (2002) based on the variability in the sequence of DF3 region. The name of a subtype consisted of the T type T4 followed by the number denoting a particular sequence of DF3 variable region. Initially 10 subtypes (T4/1 to T4/10) were recognized. Later 11 more T4 subtypes were recognized by Ledee *et al*., (2009) from T4/11 to T4/21. T4/22 and

T4/23 were reported by Abe and Kimata (2010). More subtypes were recognized after that but unfortunately the name designation was not followed and became abrupt which resulted in overlapping names. Zhao *et al*., (2010) reported seven subtypes but named then T4/22 to T4/28. Recently Magnet *et al*., (2013) reported four subtypes but named them T4/22 to T4/25. A further of four subtypes have been reported by Risler *et al*., (2013) designated as T4/31 to T4/35. A comparison of the variable regions of these subtypes is shown in Figure 2.9.

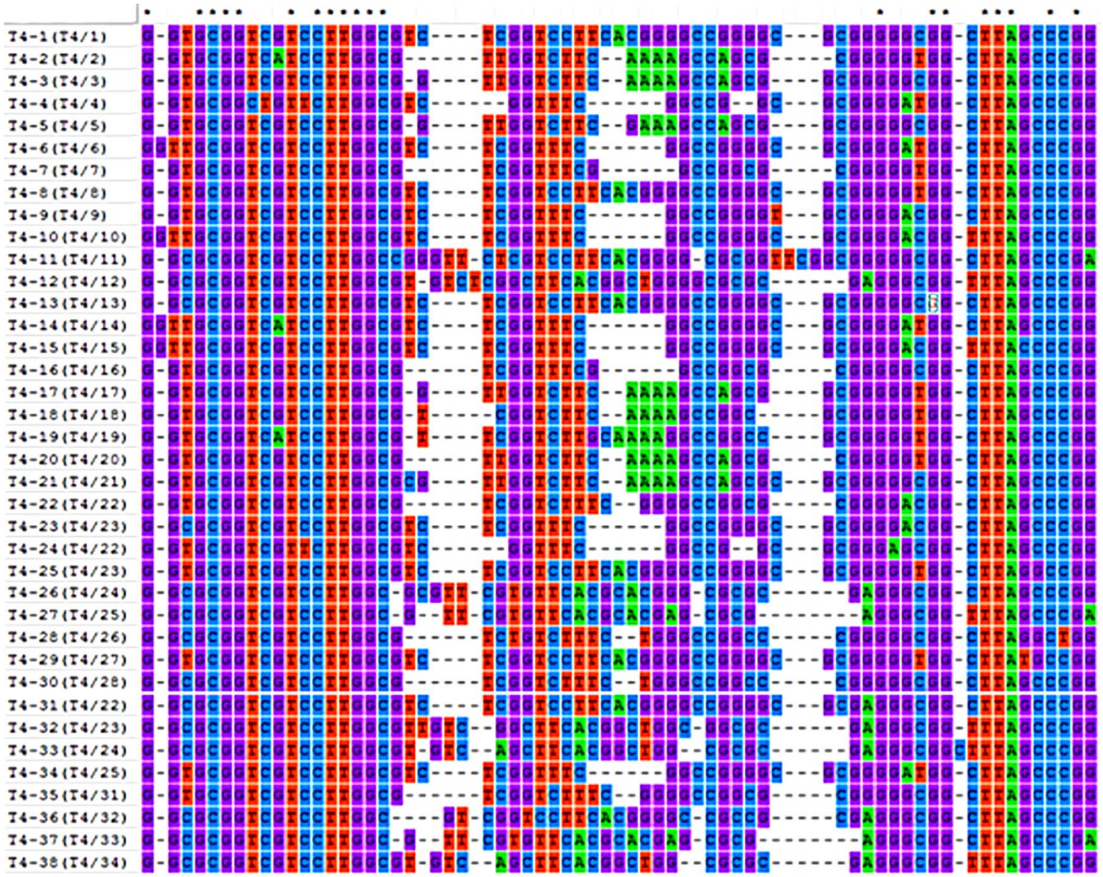


Figure 2.9: Comparison of the variable regions of the DF3 fragments of the T4 genotype of *Acanthamoeba* reported so far. The names inside parenthesis are the actual names used to designate by the workers who reported them first while the names T4-1 to T4-38 are used in this study to represent all these subtypes in chronological orders to avoid any misunderstanding in using these sequence because of the overlapping names originally assigned to them.

2.1.4. Pathogenic potential of *Acanthamoeba* isolates

For the sake of comparison, besides genotypic analysis, various parameters are used to characterize *Acanthamoeba* isolates including growth kinetics, morphological features, osmotolerance and thermotolerance. Osmotolerance is the ability to

withstand the high osmotic pressure environments like that of lacrimation due to high salinity of tears. The second important hostile factor for *Acanthamoeba* is temperature. Therefore, to survive in these kind of challenging environments *Acanthamoeba* must show omsotolerance and thermotolerance which are in general the characteristics of pathogenic *Acanthamoeba* (Khan *et al*., 2001; Khan *et al*., 2002; Walochnik *et al*., 2000b). Pathogenic *Acanthamoeba* show tolerance against both osmotolerance and thermotolerance.

2.1.5. Bacterial endosymbiotic profile of *Acanthamoeba*

In addition to above characteristics, bacterial endosymbiotic profile of *Acanthamoeba* isolates can provide further comparison of *Acanthamoeba* isolates. Endosymbiosis in simplest form mean "living together", however, the term is quite broad which includes interactions between the two organisms in a variety of forms ranging from mutual benefit (mutualism) and one-sided benefit (commensalism) to one-sided benefit with harm to the other (parasitism) (Fenchel, 1987). Unlike free-living bacteria, endosymbiotic bacteria can survive only inside eukaryotic cells (Moran, 1996). The bacterial endosymbionts of amoebae that establish long-term association have been found to belong to the Proteobacteria, the Bacteroidetes and the Chlamydiae. The Proteobacteria form the largest group and are sub-divided into alpha, beta, gamma, delta and epsilon (Madigan *et al*., 2003). Whether the presence of such bacterial endosymbionts can have any effect on preferential feeding by *Acanthamoeba* is not clear and is one of the important questions to be addressed in this chapter.

2.1.6. Objectives of the study

The main aims of the current study include the following:

2.1.6.1. Optimizing the method for isolation of *Acanthamoeba* from soil for:

- Reducing fungal contamination, accelerating adaptation of *Acanthamoeba* from xenic to axenic culture and thus minimizing overall time for isolation.
- Capability of optimized method for effective isolation of *Acanthamoeba* from soil using *E. coli*, *Enterococcus* and *Arcobacter*.

2.1.6.2. Effect of prey bacteria on the isolation of T types, subgroups and subtypes of *Acanthamoeba* from soil samples:

- Are there any feeding preferences of *Acanthamoeba* for different types of bacteria which can, in turn, affect the population of various T types/subgroups/subtypes in the environment that may be masked by using a particular type of bacteria (*E. coli*)?
- To investigate the isolates of *Acanthamoeba* on *E. coli*, *Enterococcus* and *Arcobacter* in terms of genotypic sequence analysis (diversity of sequences, genotypes, subgroups and subtypes), pathogenic profiles (thermotolerance and osmotolerance), cyst morphology and bacterial endosymbiontic profile.

2.2. Materials and Methods

2.2.1. Culture media and reagents

AX2 media for *Acanthamoeba* culture consisted of 14.3 g/L protease peptone (Fluka, Spain), 7.5 g/L yeast extract (Fluka, India), 15.4 g/L D-glucose monohydrate (Duchefa Biochemie, Netherlands), 0.51 g/L Na_2HPO_4 (Fisher Chemicals, UK) and 0.486 g/L KH_2PO_4 (Fisher Chemicals, UK). The pH was ~6.5. The PYG agar plates were prepared by adding 1.5% agar (Sigma, Portugal) (Maciver *et al*., 2013).

AX2+ media for *Acanthamoeba* culture was essentially same as AX2 media except that it contained 10 g/L of yeast extract instead of 7.5 g/L.

Vandamme media (VD***)*** for *Arcobacter* culture consisted of 10 g/L special peptone (Oxoid, England), 5 g/L Lab Lemco powder (Oxoid, England), 5 g/L yeast extract (Fluka, India), 5 g/L NaCl (Sigma Aldrich, USA), 3.25 g/L sodium succinate hexahydrate (Sigma Aldrich, Japan), 2 g/L L-glutamic acid-Na salt (Sigma Aldrich, USA) and 2.01 g/L $MgSO_4.7H_2O$ (Fisher Scientific, UK). The pH was ~6.8. The Vandamme agar plates were prepared by adding 1.5% agar (Knighton *et al*., 2013).

Lysis buffer consisted of 30 mM tris-HCl (Sigma Aldrich, USA), 5 mM EDTA (Sigma Aldrich, USA), 100 mM NaCl (Fisher Scientific, UK) and 1% SDS (Sigma Aldrich, USA) with pH 8.0 (Maciver *et al*., 2013).

Neff's saline (NS) was prepared as 10× stock solution (5 L) consisting of 6g NaCl (Fisher Scientific, USA), 0.2 g $MgSO_4.7H_2O$ (Fisher Scientific, UK), 0.2 g $CaCl_2.2H_2O$ (Oxoid, England), 7.1 g Na_2HPO_4 (Oxoid, England), 6.8 g KH_2PO_4 (Maciver *et al*., 2013).

2.2.2. Bacterial strains, culture, preservation and viable counts

Arcobacter butzleri ED-1 and *Enterococcus faecalis* were provided by Dr Bruce Ward, School of Biological Sciences, University of Edinburgh while *Escherichia coli* were provided by Dr Sutherland K Maciver, School of Biomedical Sciences, University of

Edinburgh. *Arcobacter* were cultured in VD media at 30ºC under microaerophilic conditions while *E. coli* and *Enterococcus* were cultured in LB media at 37ºC with constant shaking in an incubated shaker (Knighton *et al*., 2013; Maciver *et al*., 2013).

All the bacterial strains were cryopreserved using 20% glycerol. Liquid cultures of bacteria grown to ODs of 1.0 were used for this purpose. These were washed twice with PBS and the reconstituted with 20% glycerol in VD media (for *Arcobacter*) or LB (for *E. coli* and *Enterococcus*). Small aliquots were made in cryogenic tubes and stored at -80ºC. For revival the bacterial stocks were defrosted and washed with PBS. For *E. coli* and *Enterococcus*, washed bacteria were streaked onto LB agar plates and incubated at 37°C overnight before using these for liquid cultures. For *Arcobacter*, washed bacteria were transferred to VD media in sterile tubes and kept at 30ºC under microaerophilic conditions without shaking for 2-3 days. Before using these bacteria for the experiments the bacterial morphology and motility were monitored under microscope at 1000× to rule out any visible problems. Also the bacterial contamination was checked before sing them (Knighton *et al*., 2013).

For viable counts of bacteria ten-fold serial dilutions of bacteria were made in PBS. A few sterilized glass beads were added to the nutrient agar plate and a 100 µL aliquot was transferred onto the surface of the agar plate and shaken back and forth to spread the bacterial suspension evenly. The beads were removed and the plates were incubated to allow the bacteria to grow and form colonies.

2.2.3. *Acanthamoeba* culture, preservation and viability

The *Acanthamoeba* strains and isolates were cultured in AX2 media in cell culture flasks (75 cm^2 or 175 cm^2 depending upon the type of experiments). The propagation of cultures was made by serial passage. The old media was removed and the monolayer washed with AX2 followed by addition of further volume of AX2 to scrap the cells off the surface that were then split into new flasks. For preservation of amoebae, the cells grown to near confluence were used. The cells were scrapped off following the addition of AX2 with 5% DMSO. Aliquots of 1 mL in cryopreservation tubes were made, labelled and stored at -80ºC (John *et al*., 1994). As a good practice the cells

were not allowed to grow for indefinite number of passages, rather the cells were frozen and following revival, were used for upto 20 passages only.

The viability of *Acanthamoeba* cells was determined by the trypan blue exclusion method (Sigma-Aldrich, UK). Equal volumes (50 µL) of *Acanthamoeba* suspension and typan blue (0.4%) were mixed together and a 20 µL volume was used to charge the cell counting chamber. The total number of dead (stained) and live (unstained) cells was counted in both the chambers and the average was multiplied by the dilution factor (=2) and the correction factor (10^4) to get the number of cells per mL. The percent viability was calculated by dividing the number of live cells by the total number of cell and multiplying by 100.

2.2.4. Traditional method for isolation of *Acanthamoeba* from soil

The traditional method was only used for comparison with the optimized method while for all the other isolation experiments (second half of the chapter), only the modified method was used. Although there are slight variations in the isolation procedure, for the sake of reference the method described by Maciver *et al*., (2013) was used as representative of traditional method of isolation. Samples for traditional method were processed as shown in Figure 2.10. This involved spreading of a soil sample onto the surface of non-nutrient agar plate with some saline and leaving it overnight at RT. A small block of agar was cut out and transferred facing down onto the surface of agar plate with live *E. coli*. The plate was sealed with paraffin film and incubated at RT with daily observation until amoebae appeared. A small piece of agar was cut out at the point of amoebal growth front and transferred to another agar plate with dead *E. coli*. The process was to get a pure growth. Amoebae were then transferred to a nutrient agar plate (PYG agar) with ampicillin (50 µg/mL) and incubated until amoebal growth appeared (varied for different isolates and ranged from few days to weeks). Blocks of agar were cut out at the growth front and transferred to wells of 24-well plate along with culture media (PYG). The plate was sealed with paraffin film and incubated at RT until amoeba were adapted to the liquid culture and started to grow. The amoebae were then pooled and transferred to culture flask.

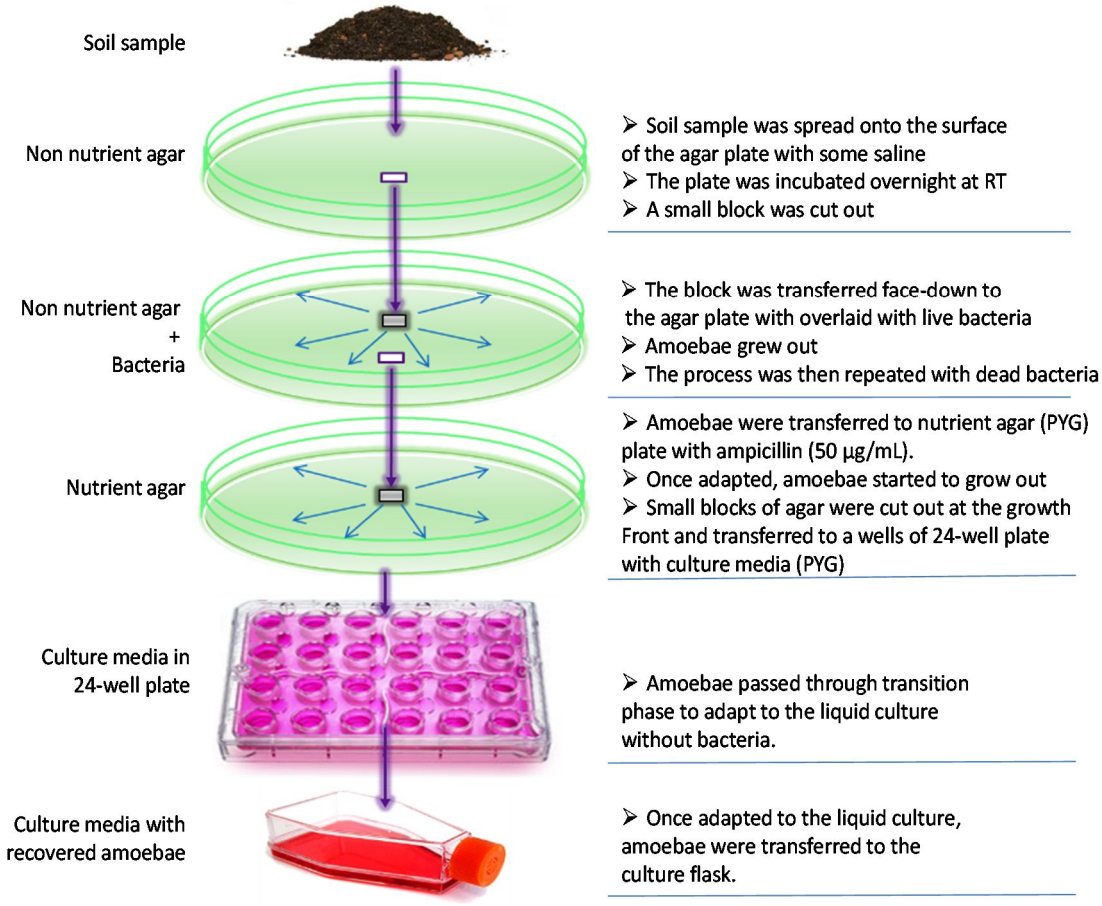


Figure 2.10: Traditional method of isolation of *Acanthamoeba* from soil using *E. coli*. This method was used as reference for comparison with the optimized method. The various steps involved are detailed in the diagram.

2.2.5. Optimization of method for isolation of *Acanthamoeba* from soil

A number of strategies were tried to find out the best conditions and to improve the isolation process for *Acanthamoeba* from soil. Various steps involved were standardized. The modified method has same main steps as the traditional method but with introduction of certain simple changes to make the isolation process smoother and faster. Figure 2.11 gives an overview of the strategy and various parameters used for the optimization process. The areas focused for optimization included bacterial killing, agar plate preparation, improving monoxenic culturing, improving growth media and improving transition from monoxenic to axenic culture. Optimization of bacterial killing is required for achieving perfect killing of microbes with the available facilities. Once optimized effectively, the sterility doesn't have to be tested every time.

Preparation of agar plates with overlaid bacteria is required to have plates with uniform thickness and spread of bacterial carpet in every batch so that amoebae have adequate bacteria to grow properly on every plate. Enrichment as the first step during monoxenic culture is intended at selectively increasing the number of amoebae before transferring to an agar plate containing bacteria. Dilution and spreading techniques are required for reducing the density of contamination (bacterial and fungal) in a given area on the plate to avoid its extensive spread. Optimization of the growth media for amoeba is essential to have better and sustainable growth over longer period of time so that amoebae can easily adapt to liquid culture and do not encyst. Easing the transition phase from non-nutrient agar plate to nutrient agar plat and then to liquid media is needed to enhance the total isolation time for amoebae

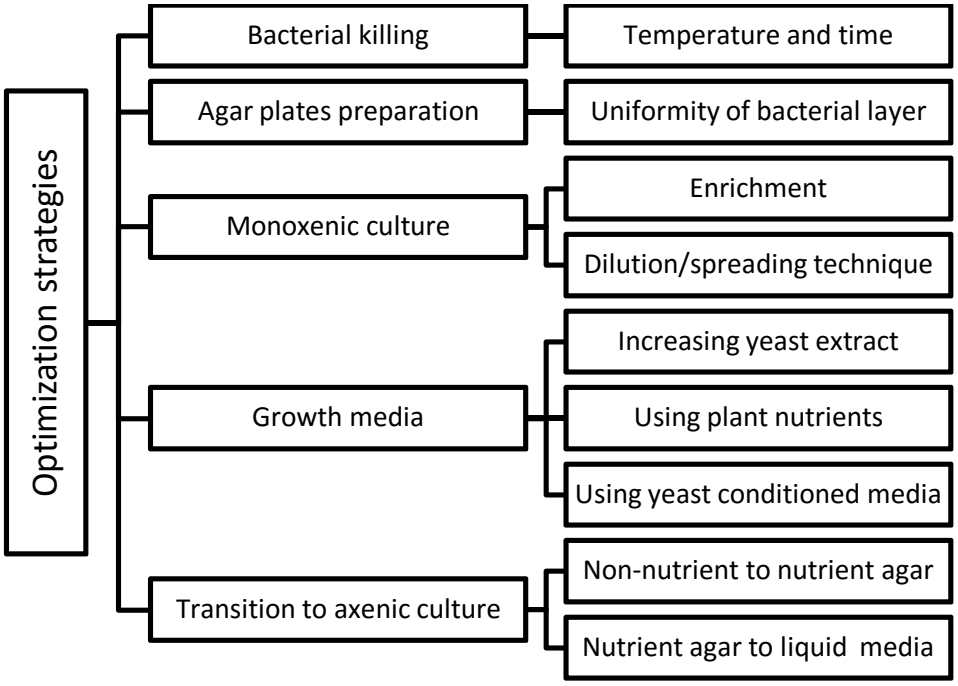


Figure 2.11: The main areas of focus during the optimization of procedures for the isolation of *Acanthamoeba* from soil. These conditions require one time standardization to obtain nearly similar results every time the procedure is repeated. Optimization of bacterial killing is aimed at achieving complete loss of viability. Standardization for the preparation of agar plates with overlaid bacteria is essential to have plates with uniform thickness having an evenly spread bacterial carpet batch after batch. During the monoxenic culture, the enrichment of amoebae during the initial phases of isolation selectively encourages the growth of amoebae to outnumber any contamination. Dilution and spreading techniques further dilute the contamination. For transition to liquid culture, the optimization of growth media for amoebae helps easing the stress of change of medium and achieve better growth for longer period of time. This reduces the total time for isolation for amoebae.

2.2.5.1. Optimizing monoxenic culturing

The various steps involved in the monoxenic culturing (bacteria-eating phase) were optimized as follows.

2.2.5.1.1. Optimizing killing process of bacteria

The conditions for the killing process were standardized in 50 mL tubes with 50 mL NS placed in an oven set at 80°C. First the time required for NS at RT (23°C) to reach the target temperature was determined by taking the core liquid temperature. The curve was plotted by taking readings for temperature at various time points. In the next step, the duration of various holding temperatures (60°C, 70°C and 80°C) required to kill the bacteria were tested. The confirmation for the complete loss of viability was made by streaking the bacterial culture on nutrient agar plates and incubating overnight to see for no colony formation.

2.2.5.1.2. Optimizing the preparation of bacterial and nutrient agar plates

This was required to eliminate variations so that all the plates are uniform in every batch. Bacteria were cultured to an OD of ~1.0 and were washed three times with PBS. Washed bacteria were reconstituted with small volume of NS. To find out the best OD suitable to make an adequate intact monolayer of bacteria, various dilutions were tested as shown in Table 2.1 to prepare agar plates. A small volume of the bacterial suspension was poured onto the surface of the plate, spread all over by swirling the plate and poured off the excess. The plates were then dried in tilting position.

Table 2.1: Various optical densities for different bacterial cultures to be used for overlaying agar plates to work out the best optical density that had enough bacteria to cover the entire surface of the 90 mm diameter agar plate evenly and to avoid batch variation.

	E. coli	***Enterococcus***	***Arcobacter***
OD (nm)	600	600	405
OD values of the culture used	1.2	1.2	1.2
	1.0	1.0	1.0
	0.8	0.8	0.8
	0.6	0.6	0.6
	0.4	0.4	0.4
	0.2	0.2	0.2

Nutrient agar consisted of 1.5% agar in AX2 media and contained combination of 10 µL of gentamicin (50 mg/mL) and 100 µL Penicillin-Streptomycin (10,000 units

penicillin and 10 mg streptomycin/mL). Each plate was prepared by pouring in 20 mL of molten nutrient agar with antibiotics at 50°C and left to dry overnight. The dried plates were overlaid with suspension of bacteria (OD_{600}=1.0 for *E. coli* and *Enterococcus*, OD_{405}=1.0 for *Arcobacter*). Excess liquid was sucked out after swirling the suspension all over the agar plate and allowed to dry in horizontal position.

2.2.5.1.3. Enrichment of *Acanthamoeba*

This was an additional step in the modified method. A small amount of soil sample was put in 1.5 mL Eppendorf tube and topped with 100 µL of live bacterial suspension (for old samples an additional step was used where soil sample was first left in Neff's saline for 1-2 days before addition of bacteria). The tube was closed and left at RT for 1-2 days if the sample was fresh or longer if the sample was old) (Figure-2.12a).

2.2.5.1.4. Minimizing contamination during monoxenic culture

This involved the dilution (with NS) and spreading of the sample to reduce the chances of contamination. For this, the samples were diluted two-fold (Figure-2.12b). Each diluted sample (50 µL) was spread onto the surface of the agar plate with an overlaid live bacterial carpet. This was done by tilting the plate and pouring sample suspension on top edge of plate and allowing the fluid to flow down to the bottom edge (Figure-2.12c). The plates were sealed with paraffin film and incubated at room RT with daily observation for the amoebal trophozoites to appear and spread (Figure-2.12d). Amoebae from the clean plate were removed and transferred to another agar plate with dead bacteria. The plates were further incubated and the process was repeated for three times to achieve a pure culture (Figure-2.12e-g).

2.2.5.2. Optimization of transition from monoxenic to axenic culturing

2.2.5.2.1. Optimizing growth media for culturing of *Acanthamoeba*

2.2.5.2.1.1. Increasing yeast extract concentration in AX2

Increased levels of yeast extract were tested for any effect on growth of *Acanthamoeba* at various concentrations i.e. 0.8, 0.9 and 1% compared to original 0.75%.

2.2.5.2.1.2. Using plant nutrients

A mixture of micronutrients used for plants growth (Miracle-Gro) was used as it contained the nutrients that are found in soil to see any positive effect on growth of

Acanthamoeba. These included nitrogen total (24.0%), ammonical nitrogen (3.5%), ureic nitrogen (20.5%), phosphorous pentoxide soluble in neutral ammonium citrate and in water (8.0%), potassium oxide (16.0%), boron (0.02%), copper (0.03%), iron (0.19%), manganese (0.05%), molybdenum (0.001%) and zinc (0.03%). The concentrations of the whole mixture used for the experiment purpose were 1, 0.1, 0.01 and 0.001%.

2.2.5.2.1.3. Using yeast conditioned media (YCM)

The yeast conditioned media (YCM) was prepared by growing yeast in AX2 media at RT with constant shaking. The cells were sedimented and the supernatant was filtered through 0.22 µm syringe filter. The testing concentrations of this YCM were 10, 1 and 0.1% in AX2. This was then used to monitor the effect on the growth of amoebae.

2.2.5.2.2. Optimizing the transition phase

Amoebae from dead agar plates were transferred to nutrient agar (AX2) plates with **dead** bacteria overlaid to help ease the transition phase (Figure-2.12h). The plates were sealed with paraffin film and left in inverted position at room temperature (23°C). The growth on these plates was quick and abundant amoebae could be seen after overnight culture. Once a heavy growth was obtained, the amoebae were washed out of the agar surface with the help of fresh AX2 media (with added antibiotics-GPS at maintenance dose i.e. 10 µL of gentamicin (50 mg/mL) solution and 100 µL of Penicillin-Streptomycin (10,000 units Penicillin, 10 mg Streptomycin/mL) per 100 mL solution. Similarly, to further ease the transition from solid agar medium to liquid medium, the recovered cells were transferred to 25 cm^2 tissue culture flask along with dead bacteria (Figure-2.12i) and monitored daily for growth. Once amoebae appeared to adapt the liquid culture, no further bacteria were added (Figure-2.12j).

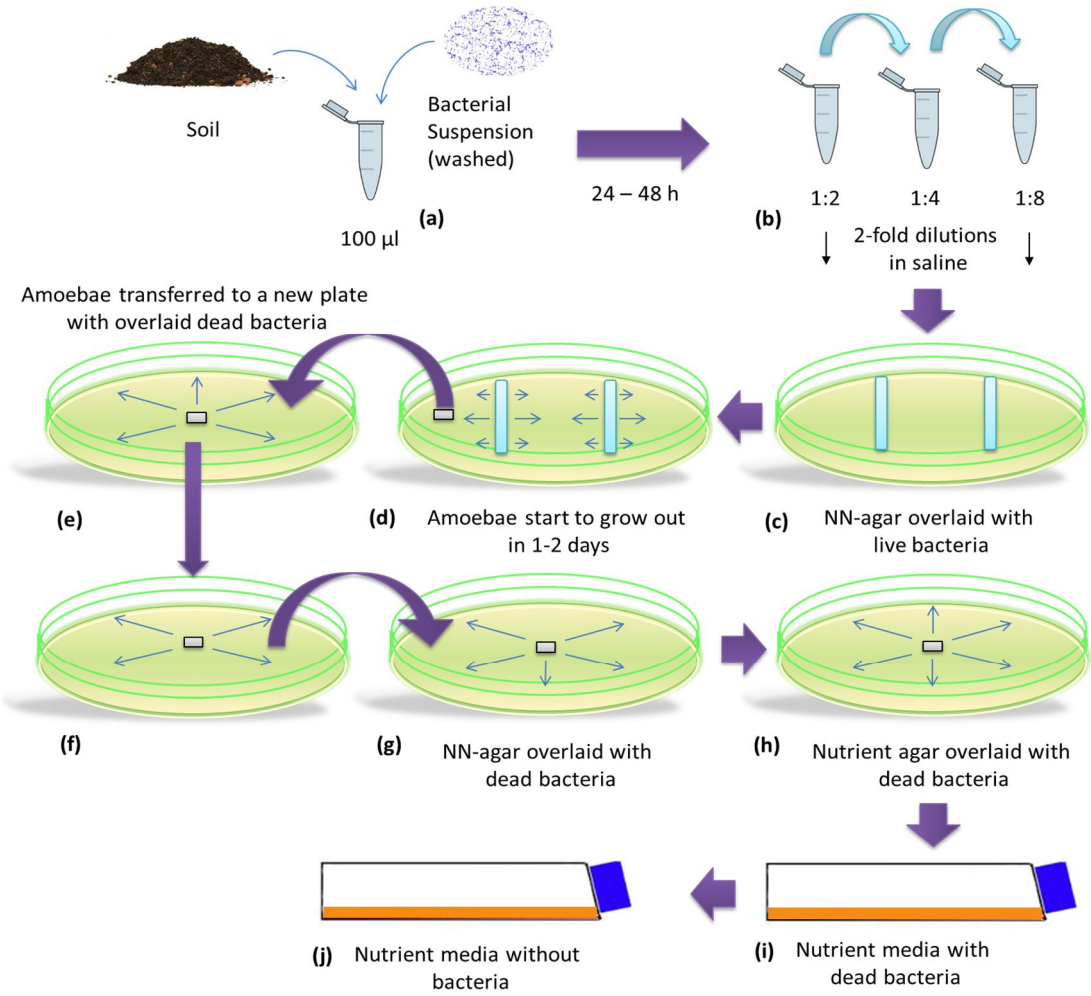


Figure 2.12: Diagrammatic representation of overall procedure of modified method for isolation of *Acanthamoeba* from soil. (a) The process starts with enrichment of amoebae in the soil sample which is achieved by adding washed bacteria to the soil in an Eppendorf tube and incubating for 24-48h. (b) In the next step dilution and spreading technique is used to minimize the level of contamination. The sample suspension is diluted 2-fold in saline. (c) The diluted suspensions are then spread edge to edge onto agar plates with bacteria. The plates are sealed and incubated. (d) Amoebae start to grow out. The plate with no or least contamination is used for further processing. A small bock was cut out along with amoebae and transferred to new agar plate with dead bacteria. (e), (f), (g) amoebae grew out and the process was repeated to achieve pure culture. (h) Amoebae were transferred to nutrient agar plate with overlaid dead bacteria to ease the transition phase. (i) Amoebae were transferred to liquid media with dead bacteria to further ease the transition phase. (j) Amoebae were transferred to liquid media without any bacteria to complete the isolation process.

2.2.6. Comparison of traditional method with modified method for isolation of *Acanthamoeba* from soil

The modified method for isolation of *Acanthamoeba* from soil was compared with the traditional method to evaluate its efficiency using *E. coli*, *Enterococcus* and *Arcobacter*. Bacteria were centrifuged and washed three times with PBS and reconstituted with NS before use. Ten soil samples were processed separately for

isolation of *Acanthamoeba* from soil on either of *E. coli*, *Enterococcus* and *Arcobacter*, making a total of 10×3=30 isolates by using both the traditional as well as optimized method in parallel. The time periods required to achieve the various isolation phases were recorded and the final assessment for any improvement in optimized method over traditional method was made on the basis of total time of isolation of *Acanthamoeba*.

2.2.7. Role of bacteria in selection of *Acanthamoeba* genotypes

To study the role of bacteria in selection of *Acanthamoeba* genotypes three different types of bacteria (*E. coli*, *Enterococcus* and *Arcobacter*) were used. The strategy used for these experiments is depicted in Figure 2.13. The isolation process of *Acanthamoeba* from soil was carried out using the optimized method. The *Acanthamoeba* isolates so obtained were compared on the basis of their genotypic analysis (18S rRNA sequence analysis), pathogenic potential (thermotolerance and osmotolerance) and bacterial endosymbiotic profile.

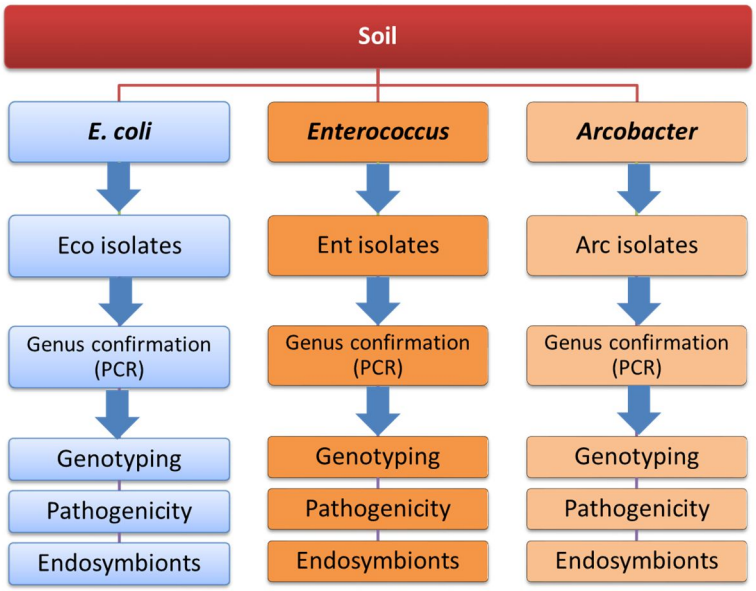


Figure 2.13: Diagrammatic representation of the experimental design for studying the effect of using different bacteria as food on the recovery of isolated *Acanthamoeba*. The soil samples were processed at the same time but separately using bacteria *E. coli*, *Enterococcus* and *Arcobacter* as the sole source of food for *Acanthamoeba*. The isolated amoebae were confirmed as Acanthamoeba by performing the genotype-specific PCR (JDP1-JDP2 primers). The isolates of *Acanthamoeba* (Eco, Ent and Arc) were compared for various parameters including genotyping by sequencing 18S rRNA genes (ASA.S1 fragment); the pathogenic potential as measured by thermotolerance and osmotolerance; and the endosymbiotic profile, studied by detecting bacterial endosymbionts using 16S rRNA sequencing.

2.2.7.1. Sample collection and processing

A total of 102 soil samples were randomly collected from various parts of England and Scotland including Burnham (10), Manchester (10), Newcastle (9), Paddington (10), Slough (7), South Kensington (8), Tilehurst (8), Victoria (12), Edinburgh (21) and North Berwick (7). Samples were brought to the laboratory and processed as soon as possible using the optimized method for isolation of *Acanthamoeba* described above. Comparative analysis of *Acanthamoeba* isolates was made to understand the role of bacteria in selection of *Acanthamoeba* genotypes. The comparison was made on the basis of genotyping analysis, pathogenic potential and bacterial endosymbiotic profile of isolates.

2.2.7.2. Genotyping analysis of *Acanthamoeba* isolates

The genotypic identification of isolated *Acanthamoeba* strains was made on the basis of 18S rRNA sequence analysis. The confirmation of genus *Acanthamoeba* and identification of isolates was carried out targeting the ASA.S1 region of 18S rRNA.

2.2.7.2.1. Extraction, storage and PCR of DNA from *Acanthamoeba*

DNA was extracted from *Acanthamoeba* using protocol described by Maciver *et al*., (2013). *Acanthamoeba* isolates that were grown in 75 cm^2 tissue culture flasks, and had reached near confluence, were used for the DNA extraction. The monolayers were washed twice with Neff's saline and the cells were scrapped off with 5 mL NS. Approximately 10^5 amoebae per Eppendorf tube were used. Each tube was centrifuged for 10 min at 1500 rpm, the supernatant was removed and 500 µL of lysis buffer was added to the obtained pellet along with 10 µL of proteinase K (2mg/ml) and incubated at 60ºC for 2h. The samples were then heated at 95ºC for 10 min to denature proteinase K. Then 500 µL of buffered phenol (pH 8.3) was introduced and the samples were centrifuged for 10 min at 13,000 rpm.

The aqueous phase from each tube was transferred to a clean tube and 500 µL of chloroform was added followed by centrifugation for 10 min at 13,000 rpm. Again the aqueous phase from each tube was transferred to another clean tube and 500 µL of chilled 2-propanol (iso-propanol) was added with 100 µL of sodium acetate (3M). The

tubes were incubated at -20ºC for 2h or overnight where immediate processing was not possible. The tubes were then centrifuged for 20 min at 13,000 rpm. All the liquid from each tube was discarded by inverting tubes and the tubes were left in inverted position so that excess liquid can flow down by itself leaving the DNA pellet behind. The samples were then washed with 70% ethanol. Finally, 30 µL water was added to resuspend the pellet. The DNA was quantified using NanoDrop.

2.2.7.2.2. PCR for genus identification and DNA sequencing for *Acanthamoeba*

For genus identification primer JDP1 and JDP2 primers were used with the sequence 5'-ggcccagatcgtttaccgtgaa-3' and 5'-tctcacaagctgctaggggagtca-3' respectively (Booton *et al*., 2002). A 50 µL volume of reaction mixture was used for each PCR reaction that comprised of 10 µL of 5× Green buffer (Promega), 4 µL $MgCl_2$ (25 mM), 5 µL each of the forward and reverse primer, 1 µL each of the dNTPs (A, G, T, C), 0.25 µL *Taq* polymerase (5 U/µL). The total volume of PCR mastermix taken for each tube was 28.25 µL to which 21.75 µL of DNA (1 ng/µL) was added to make up the total volume for each tube to be 50 µl.

The PCR conditions used included initial denaturation step at 94ºC for 3 min followed by 35 cycles starting at 94ºC for 30 sec, annealing at 55ºC for 30 sec, extension at 72ºC for 45 sec and finally 72ºC for 10 min (Maciver *et al*., 2013). The positive samples were cleaned and DNA isolated by using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer instructions. The results were visualised within an 8 µL volume of the PCR product and on 1% agarose gel. The presence of bands was confirmed before proceeding further. The extracted DNA either used immediately or where not possible was aliquoted in small quantities and stored at -20ºC until the time of use.

2.2.7.2.3. Sanger sequencing

For the DNA sequencing, Sanger sequencing was used. A 5 µL of purified DNA (4 ng/µL) and 1 µL of primer (3.2 µM) were used. The samples were sent to Genpool service (now Edinburgh Genomics) at Ashworth Laboratories, University of Edinburgh. The results were analysed further by using the software FinchTV.

2.2.7.2.4. Phylogenetic analysis

The 18S rRNA sequence for the isolates of *Acanthamoeba* was analysed to find the position of each isolate in the phylogenetic tree and assign each isolate to a genotype. The raw data obtained from the sequence analysis was carefully observed for any mistake in reading nucleotides signals. The sequences in both the directions were used to get the single consensus sequence. The primer position were located and the presence of any intron sequence was also looked for. The primer sequences were excluded before the analysis. Oligonucleotide BLAST search was made from The National Center for Biotechnology Information (NCBI) website optimized for highly similar sequences (megablast). The multiple sequence alignments were generated using MultAlin alignment tool. The best model of nucleotide substitution was worked out and the phylogenetic trees were constructed using the maximum likelihood method. The association of T types with the type of bacteria used for isolation of *Acanthamoeba* was studied by comparing the results of genotypes with the bacteria used for isolation.

2.2.7.3. Pathogenic potential of *Acanthamoeba* isolates

The temperature tolerance of the *Acanthamoeba* isolates was measured by exposing to different temperatures (40°C, 37°C and 22°C). *Acanthamoeba* cells (1×10^5) in AX2 were added to the well of a 24-well plate and the plate was incubated at the respective temperature. The plate at 22ºC served a control. The plates were sealed with paraffin film and left at respective temperatures for up to seven days. At the end of the period the plates were observed for the growth (Khan *et al*., 2001; Khan and Tareen, 2003; Lorenzo-Morales *et al*., 2005).

The osmotolerance of the isolated *Acanthamoeba* was determined by their ability to grow on 1M mannitol in AX2 media. For this purpose 1×10^5 cells in AX2 were added to the well of a 24-well plate, sealed and left at room temperature for up to seven days. The growth or no growth was noted (Lorenzo-Morales *et al*., 2005).

The photographs of the cysts and trophozoites were taken using an inverted microscope attached with camera (Canon EOS 1100D) for taking and storing photos directly into the computer.

2.2.7.4. Bacterial endosymbiotic profile of *Acanthamoeba* isolates

The isolation of DNA for bacteria endosymbionts and their detection using PCR was made as for *Acanthamoeba* (section 2.2.7.2.1.). The primers used for detection of bacterial endosymbionts were designed to target 16S rRNA signature regions, which are highly conserved within the domain Bacteria. The primers had the sequences 5'-agagtttgatymtggctcag-3' for forward and 5'-cakaaaggaggtgatcc-3' for reverse primer (Horn *et al*., 1999). A 50 µL volume of reaction mixtures was used for each PCR reaction. The details of reaction mixture and PCR conditions are same as mentioned above for *Acanthamoeba* (section 2.2.7.2.2.) except that 2.5 µL of DMSO was added per reaction tube and the annealing temperature was 58ºC with the extension at step for 2 min. Sanger sequencing and phylogenetic analysis was also used as described above for *Acanthamoeba* (sections 2.2.7.2.3./4.).

2.3. Results

This chapter deals with investigating the role of bacteria on the diversity of *Acanthamoeba* genotypes. The results for optimization of procedures for isolation of *Acanthamoeba* from soil are presented first (section 2.3.2.) followed by the main section i.e. the role of prey bacteria (*E. coli, Enterococcus* and *Arcobacter*) on the genotypic diversity of *Acanthamoeba* (section 2.3.3.).

2.3.1. Optimization of procedures for isolation of *Acanthamoeba* from soil

A number of parameters were tested in an attempt to improve the overall procedure for the isolation of *Acanthamoeba* from soil by overcoming the limitations and speeding up the procedure.

2.3.1.1. Uniformity of the procedure

To minimize the procedural variations, attempt was made to unify the basic procedures involved in the isolation of *Acanthamoeba* from soil to avoid batch variations and having uniform conditions every time.

2.3.1.1.1. Killing of bacteria

Improper killing can lead to contamination when bacteria are smeared onto the nutrient agar plates. For various bacteria the time it takes a bacterial suspension in NS at room temperature (~23°C) to reach the set temperature (80°C) differed greatly when kept in the incubator at this temperature with and without intermittent shaking (Figure 2.14). Once at the target holding temperature (80°C), the time required to completely kill the bacteria (*E. coli, Enterococcus* and *Arcobacter*) was within 10 min (Table 2.2). Therefore, the total time required for the bacteria in NS suspension at room temperature to kill the bacteria was 50+10=60 min (with intermittent shaking) and 200+10=210 min (without shaking). This ensures that the bacterial suspension is heated for enough time for complete killing and this procedure doesn't have to be repeated every time.

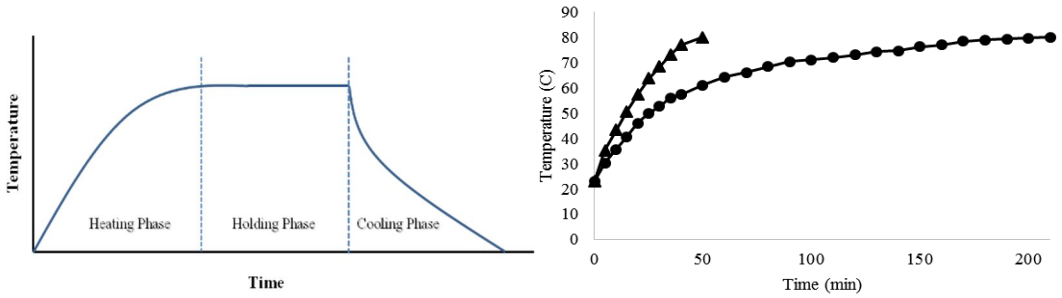


Figure 2.14: Typical temperature profile of a heat killing process of microbes in liquid (left) showing heating phase when temperature of liquid rises gradually to reach the peak to mark the holding phase where the temperature is maintained for killing process at the end of which the temperature is dropped and is called cooling phase. Figure on the right shows the temperature curve of a 50 mL saline in plastic tube at room temperature (23ºC) comparing the time it takes to reach the peak temperature (80ºC) in an oven set at 80ºC with (▲) or without (●) intermittent shaking. Therefore, it takes 50 min with shaking to achieve the peak of holding temperature (80°C).

Table 2.2: Optimization of holding temperatures (60, 70 and 80°C) of bacterial suspensions (*E. coli, Enterococcus, Arcobacter*) in saline and the times (10, 20 and 30 min) required to achieve complete loss of viability of bacteria. Except 60°C for 10 min, all other temperature-time combinations were adequate to kill all the three different types of bacteria, however, for more reliable results the holding temperature of 80°C for 10 min was used to effectively kill the bacteria.

Holding Temp (ºC)	**Time (min)**	**Viability**		
		E. coli	***Enterococcus***	***Arcobacter***
80	30	-	-	-
	20	-	-	-
	10	-	-	-
70	30	-	-	-
	20	-	-	-
	10	-	-	-
60	30	-	-	-
	20	-	-	-
	10	+	+	+

2.3.1.1.2. Preparation of live/dead bacterial agar plates

For all the three types of bacteria used (*E. coli, Enterococcus, Arcobacter*) an OD=1.0 (Table 2.3) of bacterial suspension in NS was found to be the minimum OD required to achieve a uniform and dense layer of bacterial carpet in a 90 mm diameter petri plate for overnight dried plates when smeared with bacterial suspension followed by suction of the suspension.

Table 2.3: Various ODs for different bacterial cultures to be used for overlaying agar plates to work out the best OD that has enough bacteria to make an adequately dense layer over the entire surface of the 90 mm diameter agar plate in every batch. The bacterial suspensions at OD of 1.0 were found to be adequate for this purpose for all the three types of bacteria used. The ++++ density represents a grossly dense layer of bacteria where bacteria can be seen tightly packed under the inverted microscope (×600) while the + indicates a grossly faint layer and bacteria appear widely apart under the microscope.

E. coli		***Enterococcus***		***Arcobacter***	
OD	**Layer density**	**OD**	**Layer density**	**OD**	**Layer density**
1.2	++++	1.2	++++	1.2	++++
1.0	++++	1.0	++++	1.0	++++
0.8	+++	0.8	+++	0.8	+++
0.6	++	0.6	++	0.6	++
0.4	+	0.4	+	0.4	+
0.2	+	0.2	+	0.2	+

2.3.1.2. Effect of various components on the growth of *Acanthamoeba*

Various modifications in the liquid growth medium were tested for the effect on proliferation of *Acanthamoeba* Neff (Figure 2.15). The growth of *Acanthamoeba* cells was promoted in AX2 medium with additional yeast extract, yeast-grown AX2 medium, and AX2 with 0.01% plant growth promoters. LB did not support the growth while VD could not sustain growth for longer time. Both the yeast-grown LB and VD also did not have effect on promoting growth. Very low (0.001%) and very high (0.1% and 1%) levels of plant growth promoters had no effect on growth of *Acanthamoeba* Neff cells.

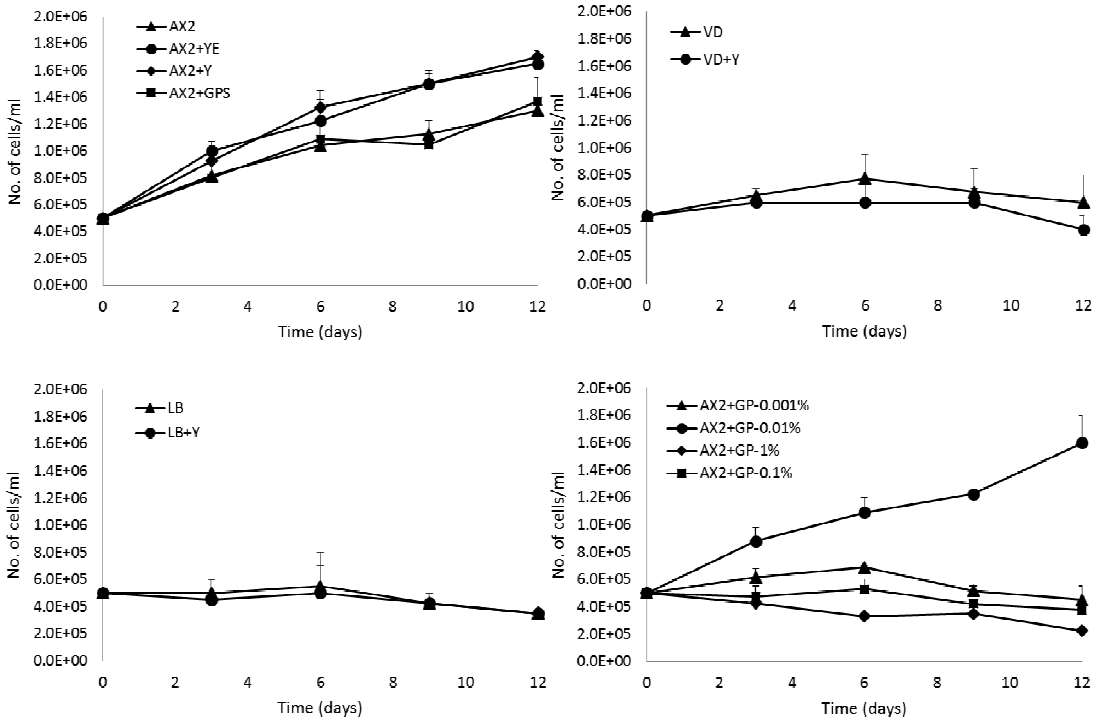


Figure 2.15: Growth curves of *Acanthamoeba* Neff in various media as indicated. AX2 was the basal medium for axenic culture of *Acanthamoeba* with 0.75% yeast extract. Various components added to this medium included YE=yeast extract (1%), Y=yeast-grown medium, GPS=gentamicin-penicillin-streptomycin ($1/10^{th}$ the normal dose i.e. at 10 µL of gentamicin 50 mg/mL solution and 100 µL of Penicillin-Streptomycin solution containing 10,000 units Penicillin and 10 mg Streptomycin/mL per 100 mL), GP=growth promoters or plant fertilizer (see description for detail) at various concentration levels. While VD=Vandamme medium used for culture of *Arcobacter*, VD+Y= yeast-grown VD medium, LB= Luria-Bertani used for routine culture of bacteria, and LB+Y=yeast-grown LB medium. While VD and LB medium appeared to have supported the growth to some extent initially but both of these didn't seem to do so for longer. Although AX2 with some modifications is widely used medium for culturing *Acanthamoeba*, addition of YE, Y and GP showed even promising results for better and sustainable growth. The graphs have been split apart for clarity. The data represents average±SE of two experiments.

2.3.1.3. Improving adaptation of *Acanthamoeba* isolates to axenic environment

Attempt to enhance the adaptation of *Acanthamoeba* isolates from monoxenic culture (single type of bacteria) to axenic culture required to be facilitated at two points of abrupt change of environment; the adaptation to axenic culture on nutrient (AX2) agar plates followed by transfer to liquid AX2 culture. The addition of dead bacteria during both of these transition phases improved the process significantly. When amoebae were transferred from simple agar plates with dead bacteria to AX2 agar plates with overlaid dead bacteria, a much rapid growth was observed after overnight incubation especially in case of Ent isolates (Figure 2.16).

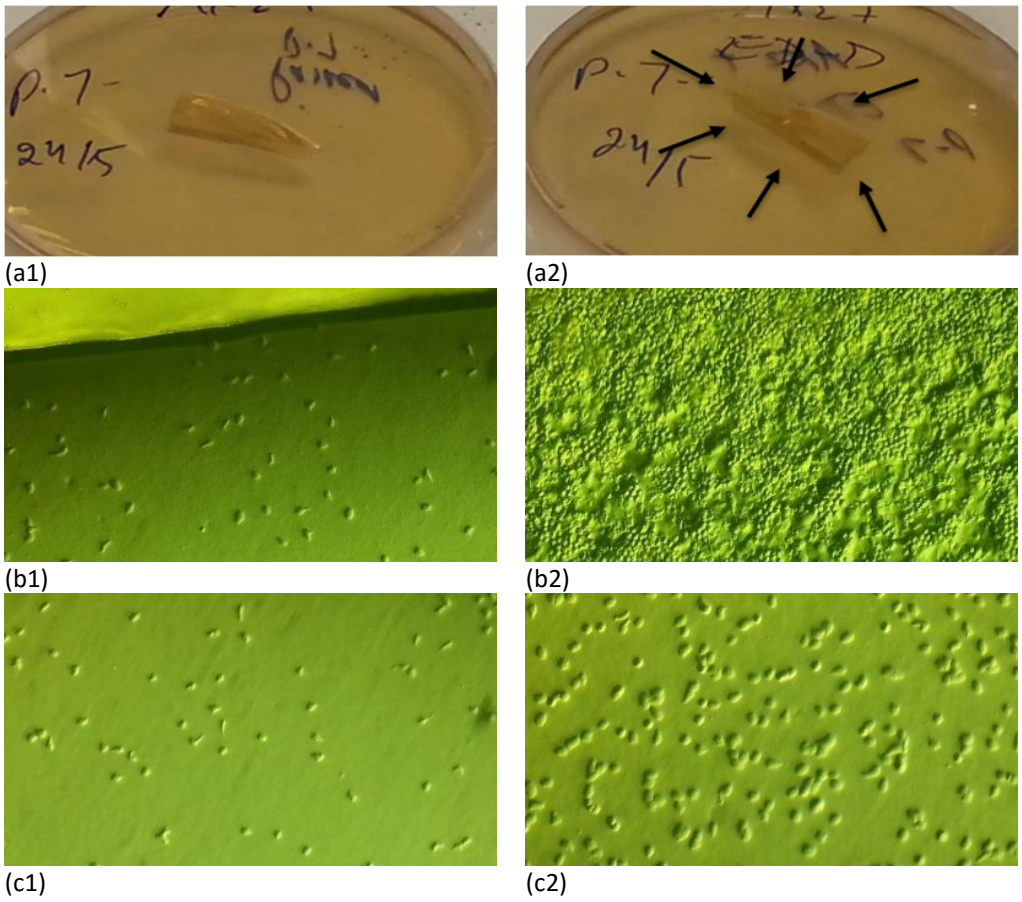


Figure 2.16: Effect of the addition of dead bacteria to the AX2 agar plate for optimized method on the rapid growth of *Acanthamoeba* as shown in overnight cultures of *Acanthamoeba* on AX2 agar plate without bacteria (control) (a1) and with bacteria (a2); excessive growth of *Acanthamoeba* can be seen in the form of dense zone (arrows) in AX2 agar plate with bacteria. The cells look bigger and healthier on plates with bacteria (b2 and c2) as compared to AX2 only plates (b1 and c1) which appear smaller probably because of the stress due to abrupt change from monoxenic to axenic culture.

2.3.2. Traditional method vs optimized method

Table 2.4 shows the results of the overall comparison of the traditional method against the optimized method for a total of 30 of Eco, Ent and Arc isolates (section 2.2.6.). Overall, there was significant different ($p<0.05$) between the time required for isolation of *Acanthamoeba* isolates at all levels (time for obtaining monoxenic culture, adaptation to axenic culture and overall time required for the whole isolation process).

Table 2.4: Comparison of traditional method and modified method in terms of time required to isolate *Acanthamoeba* from soil samples. The optimized method significantly improved the total isolation time.

***Acanthamoeba* Isolate**	**Traditional method**			**Optimized method**		
	Monoxenic culture[a] (days)	**Axenic culture[b] (days)**	**Total (days)**	**Monoxenic culture[a] (days)**	**Axenic culture[b] (days)**	**Total (days)**
Eco-E03	7	10	17	6	8	11
Eco-E05	9	12	21	7	6	13
Eco-E08	6	9	15	7	6	13
Eco-E12	7	10	17	7	7	14
Eco-P02	10	13	23	8	7	15
Eco-T01	9	10	19	8	6	14
Eco-S02	9	11	20	9	6	15
Eco-V03	9	10	19	7	8	12
Eco-NB01	10	20	30	7	6	13
Eco-NC07	7	11	18	7	8	12
Ent-E03	8	13	21	7	5	15
Ent-E05	9	15	24	7	5	15
Ent-E08	10	29	39	8	6	14
Ent-E12	14	20	34	8	5	13
Ent-P02	7	19	26	7	5	15
Ent-T01	19	18	37	8	7	15
Ent-S02	9	10	19	8	7	15
Ent-V03	8	12	20	8	5	13
Ent-NB01	8	14	22	7	7	14
Ent-NC07	7	11	18	8	5	16
Arc-E03	10	35	45	9	7	16
Arc-E05	11	16	27	8	8	16
Arc-E08	14	17	31	7	7	14
Arc-E12	9	15	24	7	6	13
Arc-P02	9	13	22	7	8	12
Arc-T01	10	10	20	7	5	12
Arc-S02	11	21	32	8	7	15
Arc-V03	11	28	39	7	6	13
Arc-NB01	12	31	43	7	5	12
Arc-NC07	13	34	47	7	6	13
Mean	9.7	16.5	26.3	7.4*	6.3*	13.7*
SE (±)	0.48	1.39	1.67	0.12	0.19	0.25

[a]extends from the onset of the monoxenic culturing to the end of three passages on dead bacteria on agar plates. It also includes any time consumed in case of contamination.
[b]extends from transferring of *Acanthamoeba* from dead bacterial agar plates to the AX2 agar plates until adaptation of cultures to liquid AX2 medium in culture flasks to near confluence.
The * indicates significant difference ($p<0.05$) by paired Student's t-test.

2.3.3. Role of bacteria (*E. coli, Enterococcus* and *Arcobacter*) on diversity of *Acanthamoeba*

Out of the total of 102 soil samples for isolation of *Acanthamoeba* isolates on *E. coli*, *Enterococcus* and *Arcobacter* carpets, the recovery of Eco, Ent and Arc isolates was 81.4%, 83.3% and 82.4% respectively; in the same order 2.8%, 2.9% and 4.9% were

contaminated while 11.8% samples in each case were found *Acanthamoeba*-negative on genus-specific (ASA.S1) PCR (Figure 2.17). Only the *Acanthamoeba*-positive amoeba isolates were processed further.

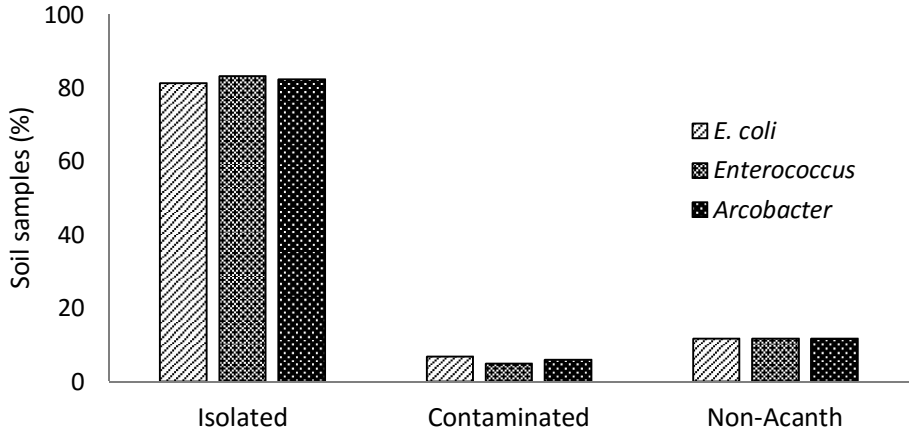


Figure 2.17: Proportional comparison of *Acanthamoeba* recovery from soil samples isolated by feeding on either *E. coli*, *Enterococcus* or *Arcobacter*, and contaminated samples that could not be processed further. In all the three cases of isolation, a good recovery of *Acanthamoeba* (>80%) was made while ~12% samples were found negative for *Acanthamoeba*.

The sequences for the three groups were also compared based on their identity with the reference sequences as well as with the Genbank sequences (Figure 2.18). The lesser the similarity, the more the divergence and hence the diversity. There was a good correlation of results between the two reference comparisons systems (reference and Genbank sequences). Among the sequences that were upto 100% match, with the references sequences or the Genbank sequences, belonged to Eco group (52.8% and 62% respectively) while the Arc isolates had the least percentage (21.7% and 43.9% respectively). However, there was a reverse trend among the sequences with upto 99% similarity i.e. 35.6% and 34.5% for Eco group vs 50.6% and 52.4% for Arc group corresponding to reference and Genbank sequences respectively. This difference was even more pronounced in case of identity of sequences with upto 98% identity to reference sequences *viz* 11.5% for Eco group vs 26.5% for Arc group.

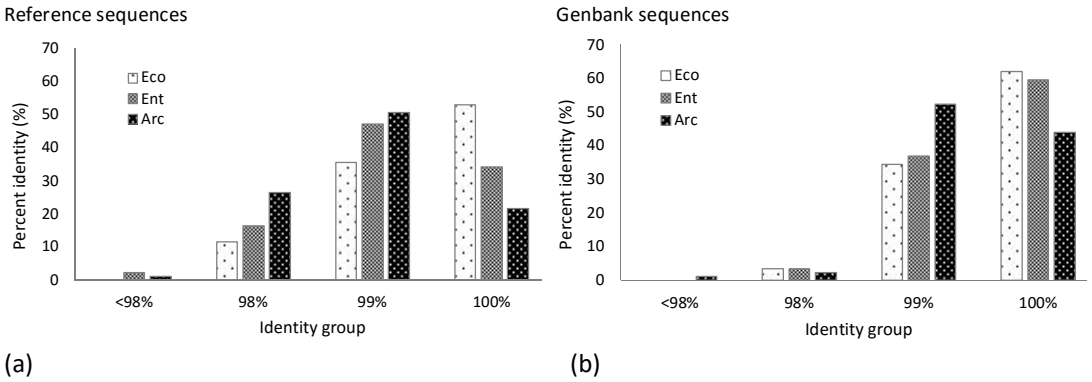


Figure 2.18: Comparison of sequence diversity of *Acanthamoeba* isolates belonging to the Eco, Ent and Arc groups in terms of similarity with the reference sequences (a) as well as with the Genbank sequences (b). Based on their percentage similarities, the sequences have been divided into four groups (100%, 99%, 98% and <98%). Interestingly, majority of the Eco isolates fall into 100% similarity category contrary to Arc and Ent isolates. However, this situation is reverse in case of lower similarity levels where most of the isolates belong to Arc and Ent isolates instead of Eco. This concludes that the Eco isolates recovered in this study are not very different from already reported sequences probably because those were also isolated on *E. coli*, however, sequences of Arc and Ent isolates show a trend of divergence from already reported sequences probably because of preferential feeding of *Acanthamoeba* on these bacteria. Total number of *Acanthamoeba* isolates were 83, 85 and 84 for Eco, Ent and Arc, respectively.

2.3.3.1. *Acanthamoeba* isolates on *E. coli*

The detailed account of the *Acanthamoeba* isolates on *E. coli* is given in Appendix-IA. T types T2, T4, T11 and T13 were isolated while none of the isolates had unique sequence. T4 was the major type isolated *viz* 89.2% (74/83) followed by T2 *viz* 6.0% (5/83), T11 and T13 both at 2.4% (2/83) (Figure 2.19).

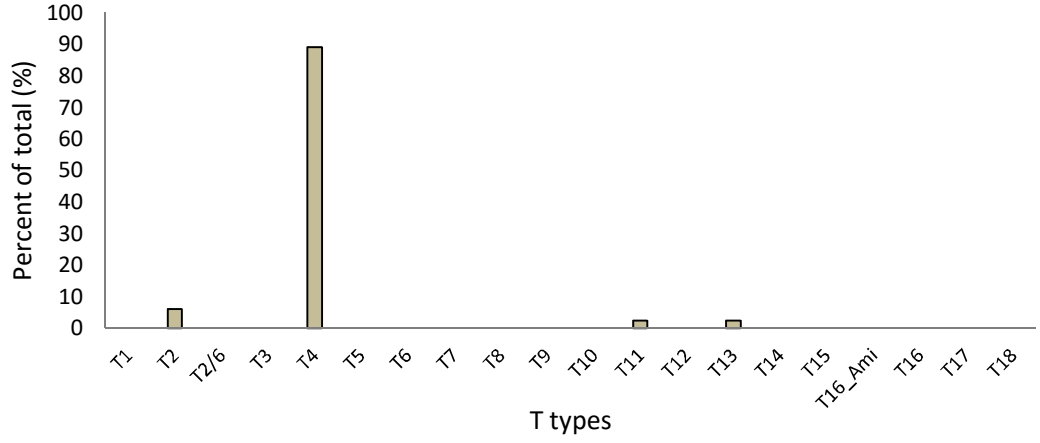


Figure 2.19: *Acanthamoeba* T types isolated from soil samples processed on *E.coli*. Overall, T2 (6.0%), T4 (89.2%), T11 (2.4%) and T13 (2.4%) T types were recovered. Thus T4 was the major T type as it is usually seen. Total number of Eco isolates was 83.

2.3.3.1.1. T2 isolates

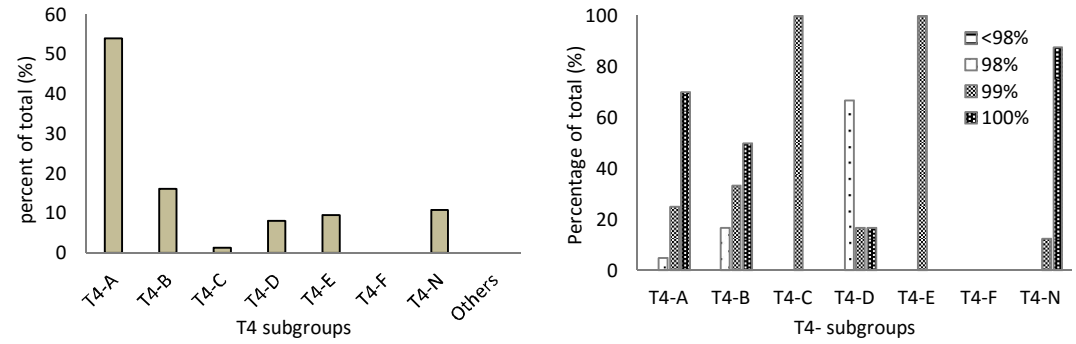
On the basis of the ASA.S1 segment sequence similarity, the isolates were unambiguously 99-100% similar to the T2 sequences of the reference sequences as well as to the Genbank database sequences. These sequences were, therefore, presented little divergence and were closely similar to already known sequences.

2.3.3.1.2. T4 isolates

Overall the T4 isolates had 98-100% similarity with the reference sequences as well as with the Genbank database sequences indicating less divergence from the already reported sequences. The T4 sequences were further analysed down to subgroup and subtype levels for a detailed insight and comparison with Ent and Arc isolates.

2.3.3.1.2.1. T4 subgroups

Among the seven recognized subgroups of T4, six were identified (T4-A, -B, -C, -D, -E and -N) whereas T4-A was the most abundant (40/74=54.1%) followed by T4-B (12/74=16.2%), T4-N (8/74=10.8%), T4-E (7/74=9.5%), T4-D (6/74=8.1%) and T4-C (1/74=1.3%). No unusual sequence was found (Figure 2.20a). All the sequences were in a narrow similarity range of 98-100%. T4-N had the least divergence with 87.5% sequences upto 100% similar to reference sequences while T4-D showed the greatest divergence with 66.7% sequences at upto 98% similarity (Figure 2.20b).



(a) (b)

Figure 2.20: (a) *Acanthamoeba* T4 subgroups isolated from soil samples processed on *E.coli*. T4-A was the most abundant (40/74=54.1%) while T4-C (1/74=1.3%) was the least frequent. (b) Comparison of percent similarity to the reference sequences among various subgroups of T4 types isolated on *E. coli*. T4-N was the least divergent (87.5% sequences upto 100% similarity) while T4-D had the greatest divergence (66.7% sequences at 98% similarity). Total number of T4 sequences was 74.

2.3.3.1.2.2. T4 subtypes

Out of the total of 39 reference T4 subtypes (T4-1 to T4-39), the isolated T4 genotypes were found to belong to one of the 11 subtypes (T4-1, -8, -12, -16, -20, -23, -26, -31, -32, -35 and -36) with T4-36 being the most abundant while T4-20/26 being the least abundant (33.8% and 1.4%) respectively. The percent similarity ranged from 96-100% while the 9.5% isolates were unique being in low range (82-88% similarity) and were, therefore, categorized as "others" (Figure 2.21).

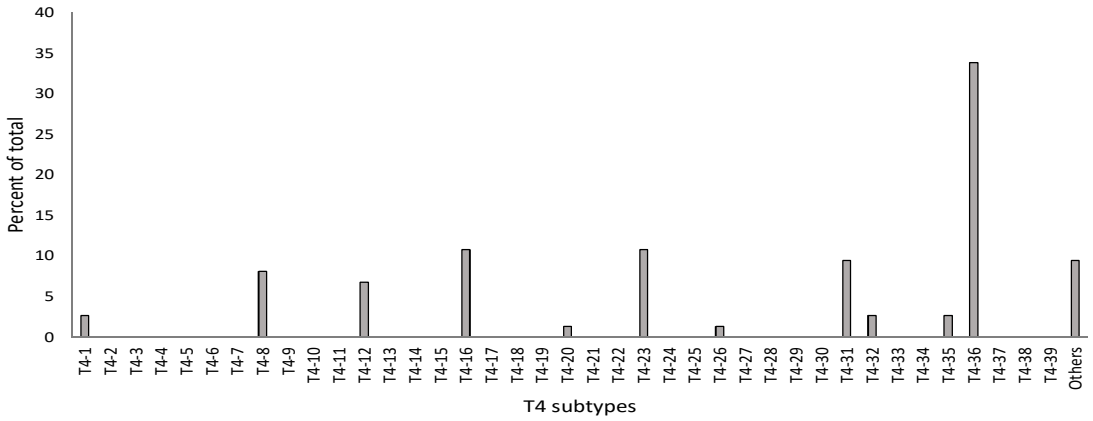


Figure 2.21: Percentage distribution of T4 types isolated on *E. coli* into T4 subtypes (T4-1 to T4-39). T4-36 was the most abundant while T4-20/26 were the least abundant (33.8% and 1.4%).

2.3.3.1.3. T11 isolates

T11 sequences were 99% similar to the reference sequences while 100% identical to the Genbank database sequence (KJ801938.1).

2.3.3.1.4. T13 isolates

T13 isolates had 98% similarity to the reference sequences while they showed 98-100 similarity to the Genbank sequences.

2.3.3.1.5. Cyst shapes

Most of the *Acanthamoeba* isolates on *E. coli* had star-shaped cysts (Figure 2.22). These were then used to compare with the cysts of Ent and Arc isolates for any similarities or differences.

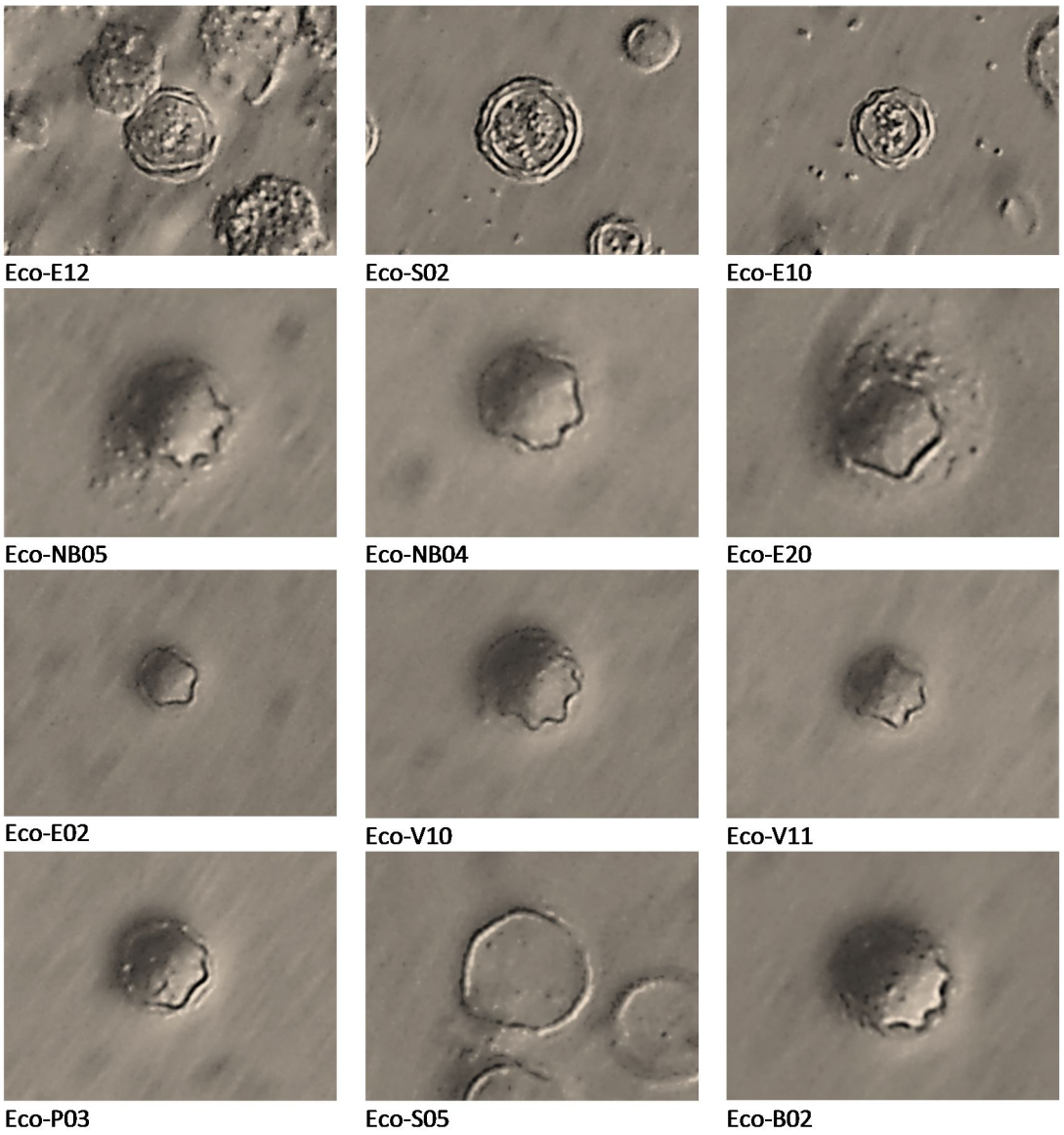


Figure 2.22: Morphological features of cysts of soil isolates of *Acanthamoeba* isolated on *E. coli.*

2.3.3.2. *Acanthamoeba* isolates on *Enterococcus*

The detailed account of *Acanthamoeba* isolates on *Enterococcus* is given in Appendix-IB. *Acanthamoeba* isolated on *Enterococcus* showed comparatively less diversity as only T4 (95.1%) and T16 (3.7%) were identified, while 1.2% isolates were not a good match of any of the reference sequences (Figure 2.23). Overall, the sequences had 96-100% similarity with the reference sequences while 98-100% similarity with the Genbank database sequences.

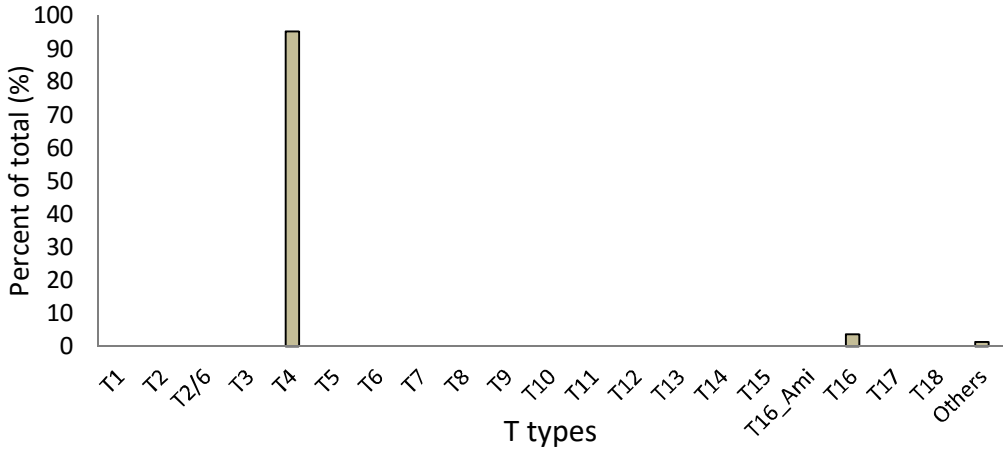


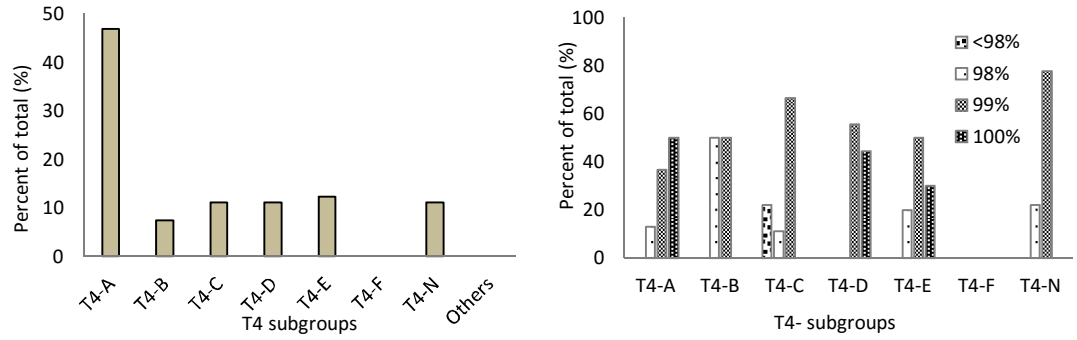
Figure 2.23: *Acanthamoeba* T types isolated from soil samples processed on *Enterococcus*. Overall, less T types diversity was observed as compared with Eco isolates as only T4 (95.1%) and T16 (3.7%) Types were recovered while 1.2% had different 18S rRNA sequence which was intermediate between T13 and T16.

2.3.3.2.1. T4 isolates

Overall the T4 isolates had 96-100% similarity with the reference sequences and 98-100% similarity with the Genbank database sequences.

2.3.3.2.1.1. T4 subgroups

Six subgroups of T4 were identified including T4-A, -B, -C, -D, -E and -N. T4-A was the most abundant (47.0%) as in Eco isolates while T4-B was the least (7.4%) abundant (Figure 2.24) unlike Eco isolates where T4-C was the least abundant. Majority of the sequences were in 98-100% similarity range to the reference sequences. The most divergent sequences as compared to the reference sequences were for T4-C with 22.2% sequences that were even below 98% similarity level. The least sequence diversity was shown by T4-D isolates who had all the sequences at 99-100% similarity with the reference sequences.



(a) (b)

Figure 2.24: (a) *Acanthamoeba* T4 subgroups isolated from soil samples processed on *Enterococcus*. All the T4 subgroups except T4-F were recovered with T4-A (47.0%) being the most while T4-B (7.4%) the least abundant. (b) Comparison of percent similarity to the reference sequences among various subgroups of T4 types isolated on *Enterococcus*. T4-C showed the most divergent sequences as compared to the reference sequences with 22.2% sequences even below 98% similarity level. On the other hand T4-D isolates were found to have the least sequence diversity as all the sequences had 99-100% similarity with the reference sequences.

2.3.3.2.1.2. T4 subtypes

The isolated T4 types belonged to the T4 subtypes T4-7, -8, -12, -16, -22, -23, -26, -27, -31, -35 and -36. T4-36 (19.8%) was the most while T4-7 was the least abundant (1.2%) subtype of T4. Percent similarity ranged from 95-100% while 6.2% isolates were below 95% similarity range and were categorized as "others" (Figure 2.25).

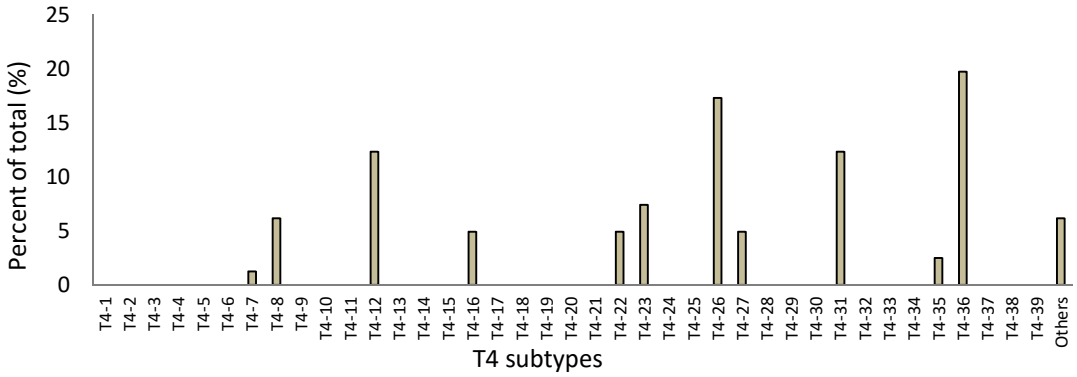


Figure 2.25: Percentage distribution of T4 types isolated on *Enterococcus* into T4 subtypes (T4-1 to T4-39). Overall, 11 different subtypes were detected while 6.2% isolates had unique sequences. T4-36 emerged as the most abundant while T4-7 was the least abundant subtype.

2.3.3.2.2. T16 isolates

All the three T16 isolates were in 100% similar range with both the reference and the Genbank sequences.

2.3.3.2.3. T13/T16 isolate

One of the isolates was no-perfect match for any of the reference sequences. The nearest matches were T16 (97%) and T13 (96%) as evident by the sequence alignment as well (Figure 2.26). A full-length sequence would be required to know if this sequence represents a new T type.

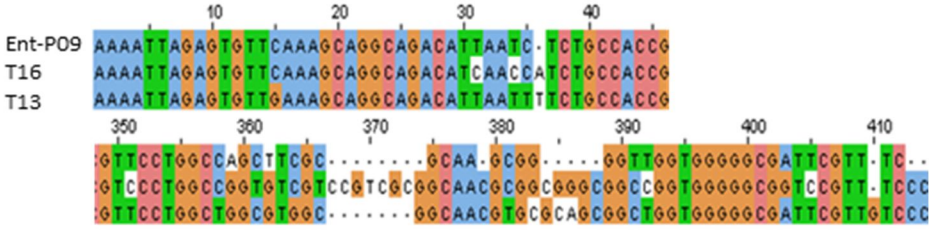


Figure 2.26: Sequence alignment of the *Acanthamoeba* isolate Ent-P09 with the nearest matching T16 and T13 reference sequences showing mismatched sites throughout the ASA.S1 fragments of 18S rRNA. The sequence doesn't show perfect similarly with either of the T13 (AF132134) or T16 (AY026245). Further investigation may reveal more information about this isolate.

2.3.3.2.4. Cyst shapes

Unlike Eco isolates, the Ent isolates of *Acanthamoeba* did not have star-shaped cysts. Rather they were mostly rounded or slightly wrinkled (Figure 2.27) despite the fact that T4 was the major T type. This indicates that the isolated amoebae in Eco and Ent groups were also different on morphological basis.

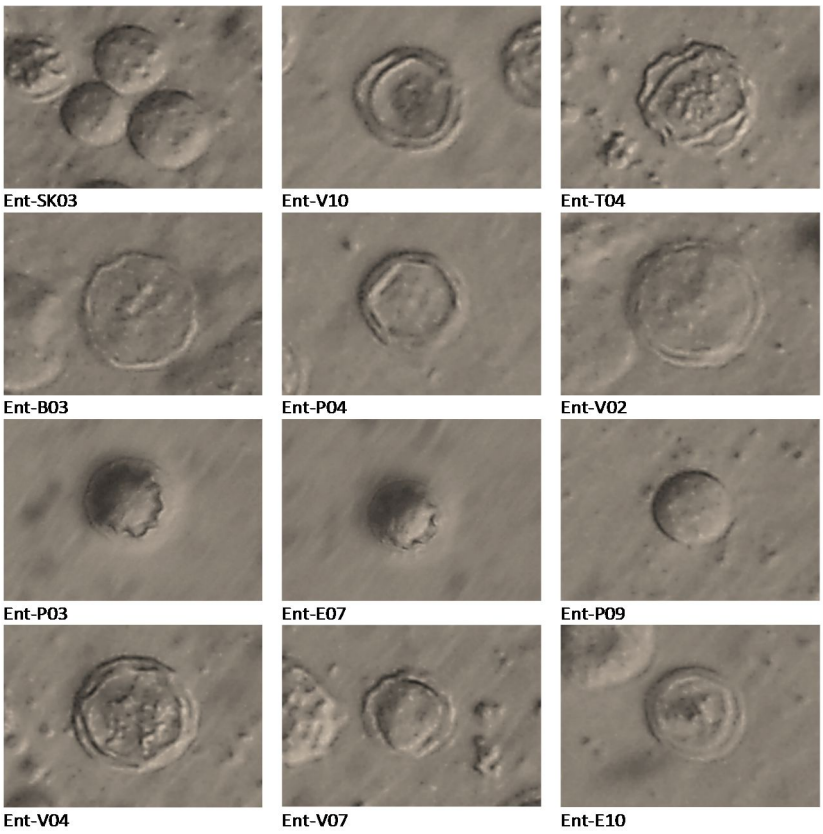


Figure 2.27: Morphological features of cysts of soil isolates of *Acanthamoeba* isolated on *Enterococcus*.

2.3.3.3. *Acanthamoeba* isolates on *Arcobacter*

The detailed account of *Acanthamoeba* isolates on *Arcobacter* is given in Appendix-IC. T types T2, T2/6, T4 and T13 were isolated while two isolates had relatively different sequences and appeared to be fall between T13 and T16. T2 (14.3%) was the main T type after T4 (78.6%) (Figure 2.28). The Arc isolates showed greater diversity of T types of *Acanthamoeba* isolates than Eco or Ent isolates as manifested by the recovery of T2, T2/6, T4, T13 and intermediate sequences. Only 78.6% T4 isolates were recovered in case of Arc isolates as compared to 95.3% of Ent and 89.2% of Eco isolates. Emergence of T types in Arc isolates but not in Eco isolates is interesting as well as important.

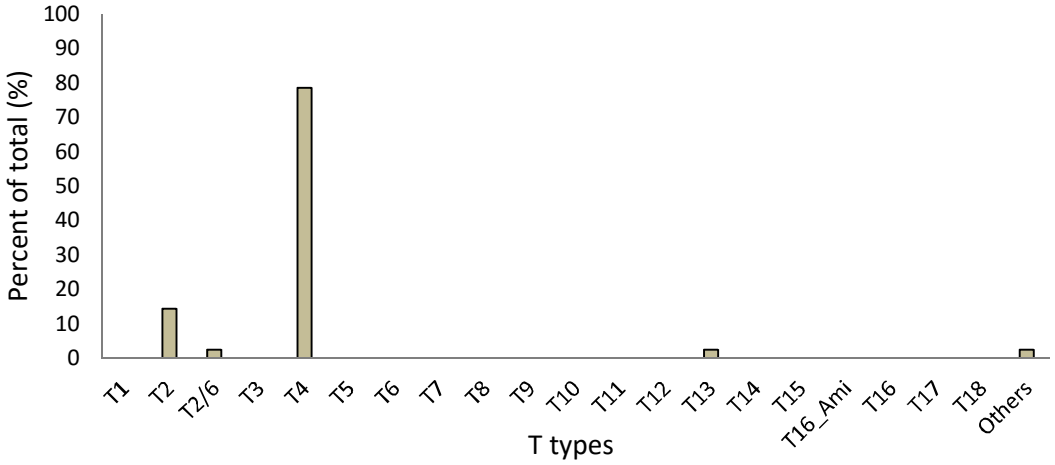


Figure 2.28: *Acanthamoeba* T types isolated from soil samples processed on *Arcobacter*.

2.3.3.3.1. T2 isolates

The sequences of the T2 isolates were 94-100% similar to the T2 sequences of the reference sequences and 98-100% similar to the Genbank database sequences.

2.3.3.3.2. T2/T6 isolates

The two T2/T6 isolates were 98% and 99% identical to reference sequences and Genbank sequences respectively.

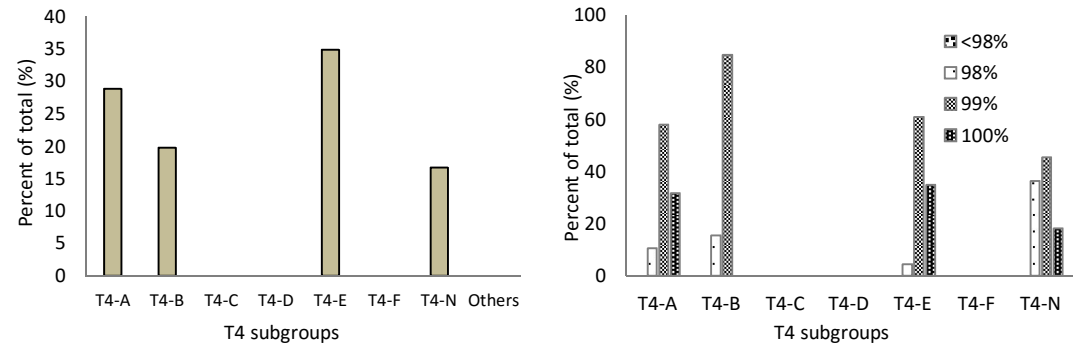
2.3.3.3.3. T4 isolates

Overall the T4 isolates had 98-100% similarity with the reference sequences and 99-100% similarity with the Genbank database sequences.

2.3.3.3.3.1. T4 subgroups

Only four subgroups of T4 were identified including T4-A, -B, -E and -N. Unlike *E. coli* and *Enterococcus* isolates, the *Arcobacter* isolates of *Acanthamoeba* had T4-E as the most abundant (34.8%) T4 subgroup followed by T4-A (28.8%), T4-B (19.7%) and T4-N (16.7%) (Figure 2.29a). All the sequences were in a similarity range of 98-100%. The greatest similarity with the reference sequences was shown by T4-E with 34.7% sequences 100% similarity while T4-N showed the greatest divergence with 36.4% sequences at 98% similarity (Figure 2.29b). This was unlike Eco isolates where none of T4-N isolate was at 98% similarity and Ent isolates where only 22.2% isolates

were at 98% similarity. Overall, T4-B showed the greatest diversity of sequences in terms of divergence from the references sequences as no sequence was 100% similar to reference sequences and all the sequences were in 98-99% similarity range. While T4-E showed the least diversity as most of the sequences were in 99-100% similarity range.



(a) (b)

Figure 2.29: (a) T4 subgroups of *Acanthamoeba* isolated from soil samples processed on *Arcobacter*. Unlike Eco and Ent isolates, Arc isolates lacked T4-C and T4-D subgroups of T4 types. (b) Comparison of percent similarity of to the reference sequences among various subgroups of T4 types isolated on *Arcobacter*.

2.3.3.3.3.2. T4 subtypes

The isolated T4 types were found to belong to subtypes T4-12, -13, -22, -23, -31, -34, -35 and -36. Unlike isolates of *E. coli* and *Enterococcus*, the *Arcobacter* isolates had T4-12 as the most abundant subtype (31.8%). The percent similarity ranged from 95-100% while the 13.6% isolates were in low range of 88-92% similarity and were categorized as "others" which constituted a major group (Figure 2.30). Unlike Eco isolates T4-1, T4-8, T4-16, T4-20, T4-26 and T4-32 were absent in Arc isolates but T4-13, T4-22 and T4-34 were found only in Arc isolates. This shows various T4 subtypes might have preferential feeding upon different types of prey bacteria.

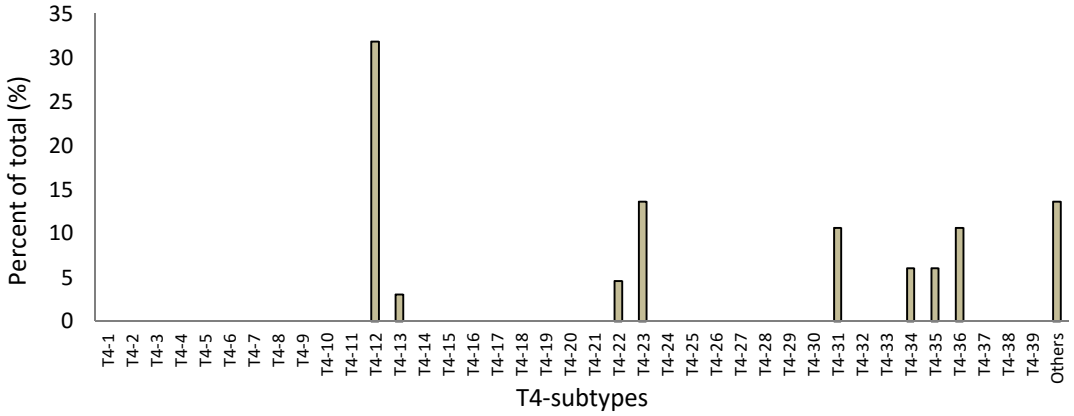


Figure 2.30: Percentage distribution of T4 types isolated on *Arcobacter* into T4 subtypes (T4-1 to T4-39).

2.3.3.3.4. T13 and T13/16 isolates

These sequences were greatly divergent as they had 97-98% similarity with the reference as well as Genbank sequences (Figure 2.31). *E. coli* were unable to isolate the intermediate T13/T16 isolates. Even the sequences of T13 were more divergent than those of Eco isolates that were 98-100% similar to Genbank sequences. This indicates that *E. coli* in this case were not only unable to isolate T13/16 but also they missed the more divergent T13 isolates. This also indicates that probably there are many different versions of a single T types at various sequence divergence levels that cannot be effectively isolated using a single bacterium type.

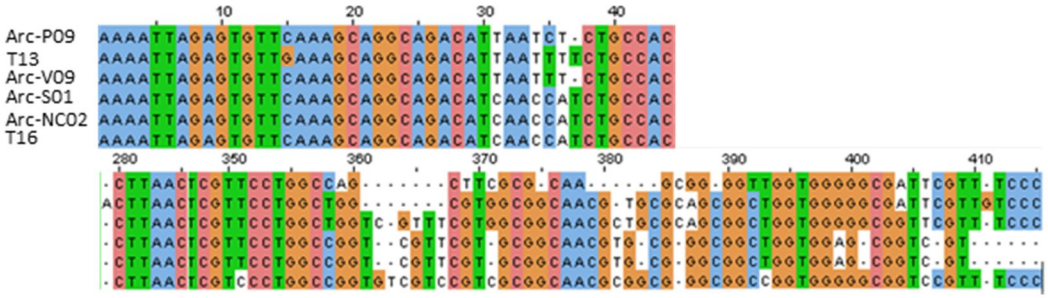


Figure 2.31: Comparative sequence alignment of ASA.S1 fragments of *Acanthamoeba* isolates Arc-P09 (T13), Arc-V09 (T13), Arc-S01 (T13/16) and Arc-NC02 (T13/16) with T13 (AF132134) and T16 (AY026245) reference sequences showing mismatched sites throughout the ASA.S1 fragments.

2.3.3.3.5. Cyst shapes

Unlike Eco isolates, the Arc isolates of *Acanthamoeba* did not have star-shaped cysts. Rather they were mostly rounded or slightly wrinkled (Figure 2.32).

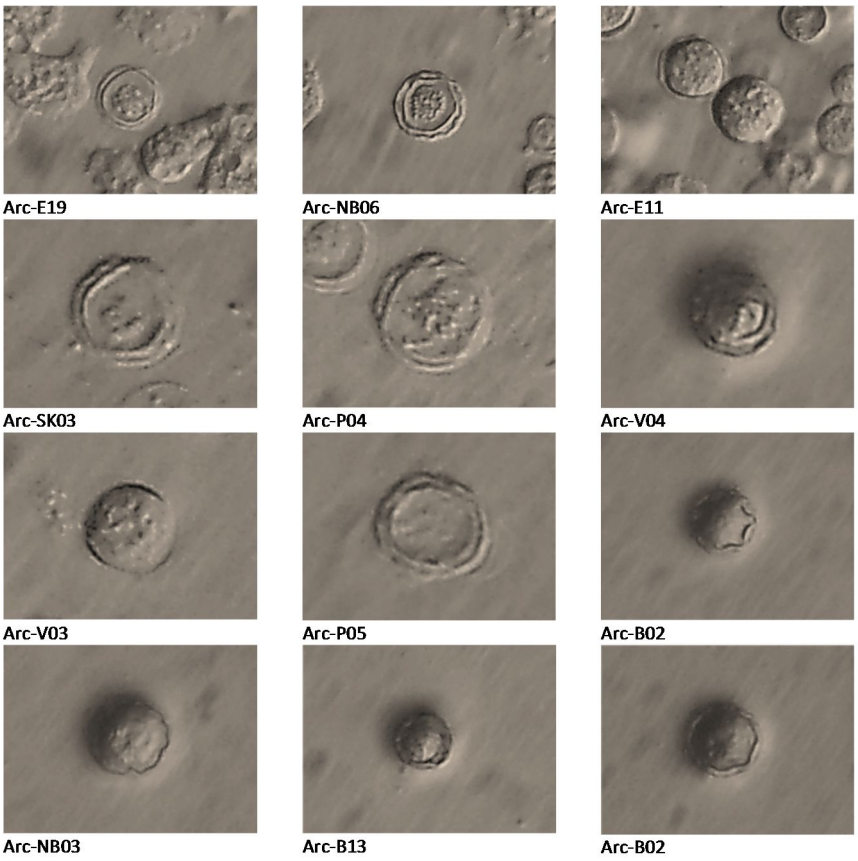


Figure 2.32: Morphological features of cysts of soil isolates of *Acanthamoeba* isolated on *Arcobacter.* Although there were few star-shaped cysts but overall the Arc cysts were different morphologically from the star-shaped cysts found in case of Eco isolates.

The overall phylogenetic tree for the important isolates of Eco, Ent and Arc isolates is shown in Figure 2.33 along with reference sequence to establish the positions of the isolates in relation to the reference sequences. The greater divergence of Arc isolates compared to Eco isolates is clearly evident. Among the T2 isolates, the Eco-T02, Eco-S07, Arc-P06 and Arc-E09 were quite similar to T2 reference sequences but majority of Arc isolates formed a relatively distinct group close to T2 comprising of Arc-T01, Arc-V13, Arc-B08, Arc-NB03, Arc-T02, Arc-S07, Eco-S05, Arc-SK07, while two of the Arc isolates formed even more divergent and isolated group comprising of Arc-P11 and Arc-T08. The divergence of Arc-S01 (T13/16) and Arc-NC02 (T13/16) isolates was also evident from the tree as these formed an isolated group between T13 and T16. Again the greatest divergence of sequences was shown by Arc isolates.

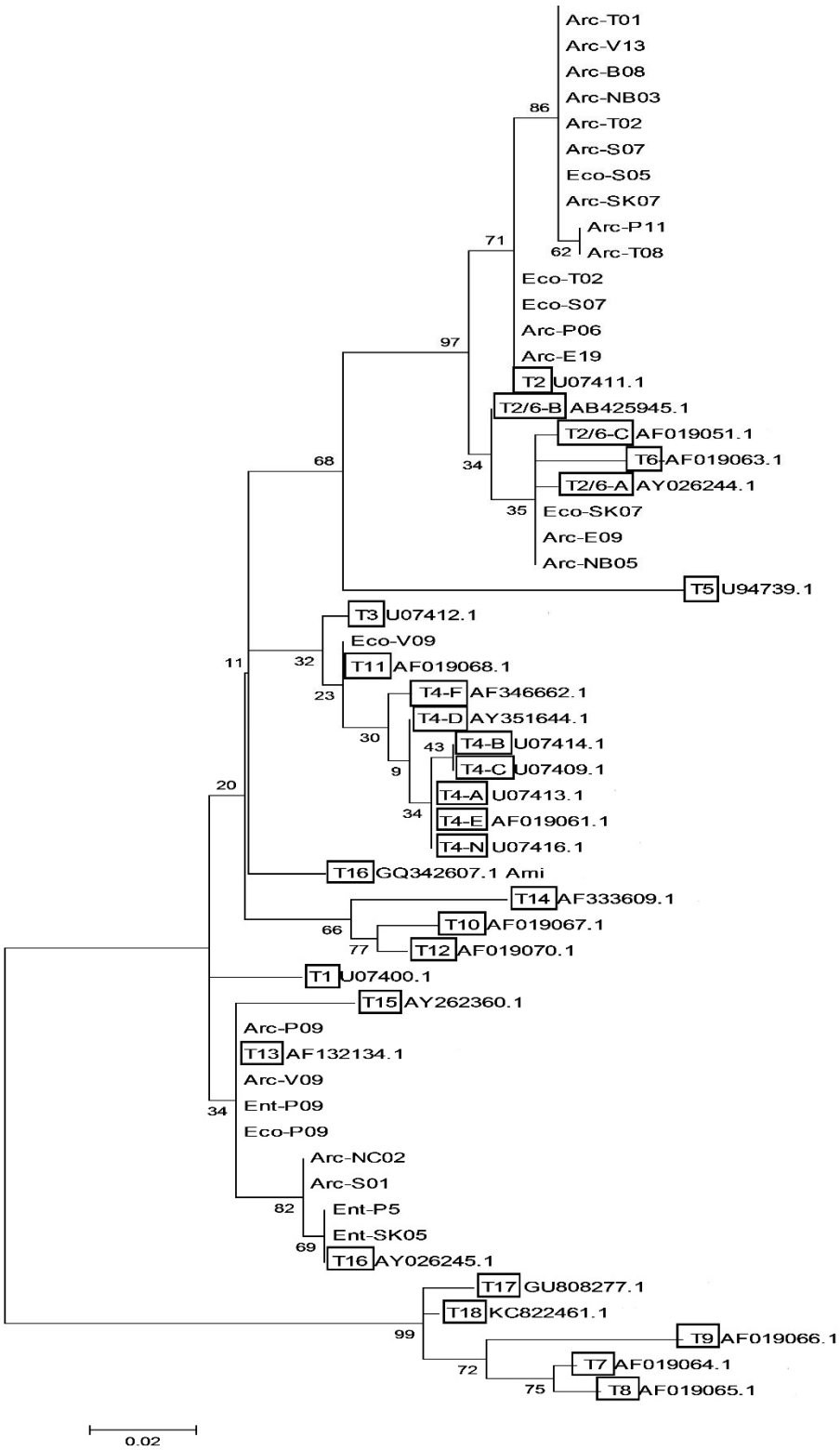


Figure 2.33: Maximum likelihood phylogenetic tree of *Acanthamoeba* reference T types and the *Acanthamoeba* isolates (this study) showing relative position of isolates based on ASA.S1 fragment sequences. A bootstrap value of 500 was used. The tree was generated using MEGA 6.0. The tree provides information about the position of various Eco, Ent and Arc isolates from this study. It also confirms the findings of the sequences matching with the reference sequences based on percent similarity of sequences.

2.3.3.4. Pathogenic potential of *Acanthamoeba* isolates

The pathogenic potential of the *Acanthamoeba* isolates was estimated based on the ability of individual isolates to withstand higher temperatures and survive the high osmotic pressure environment created by 1M mannitol. The detailed results of thermotolerance and osmotolerance are presents in Appendix-IIA, -IIB and –IIC.

Thermotolerance and osmotolerance were not very useful in differentiating the three isolate groups (Eco, Ent and Arc) which might be because of environmental nature of these samples. At 41°C there was almost no growth isolate while at 37°C majority of isolates in the three groups had only poor growth (Figure 2.34). None of the isolates showed clear osmotolerance to 1M mannitol. This might be because of the environmental nature of the samples.

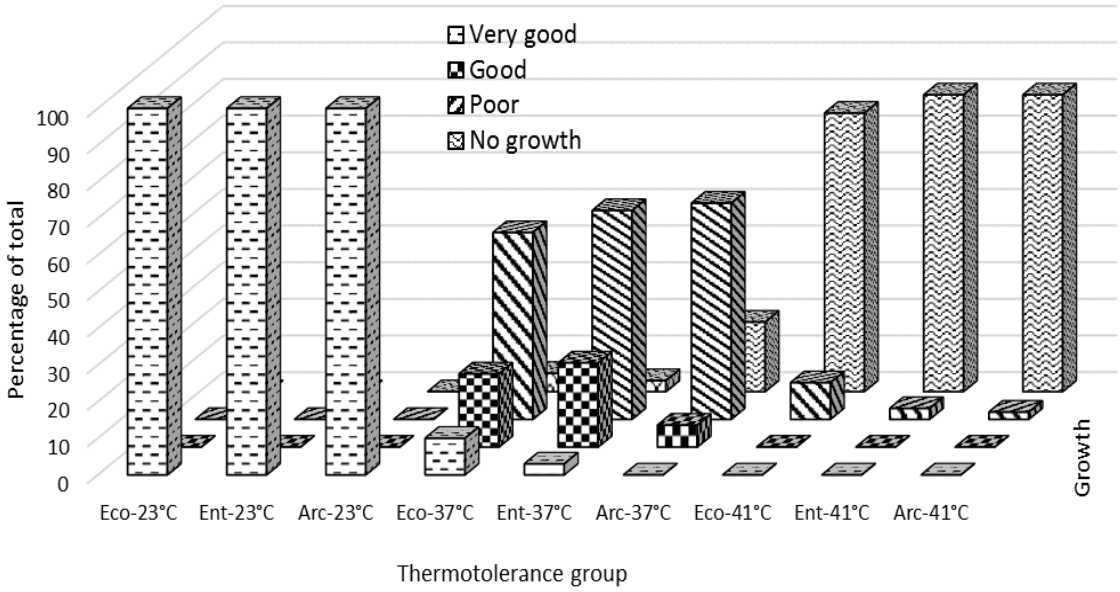


Figure 2.34: A three-dimensional comparison of various thermotolerance groups of the recovered Eco, Ent and Arc *Acanthamoeba* isolates, based on growth at 23ºC, 37ºC and 41ºC, and ranked as very good (VG), good (G), poor (P) or none (N). At 23°C all of the three types of *Acanthamoeba* isolates (Eco, Ent and Arc) had very good growth in medium in contract to 41°C where almost none of the isolates had shown any growth. The difference among the three types of isolates were slightly clear at 37°C where overall Arc isolates showed less tolerance to temperature as compared to Eco and Ent isolates.

2.3.3.5. Bacterial endosymbiontic profile of *Acanthamoeba* isolates

The detailed account of the bacterial endosymbionts in *Acanthamoeba* isolates is given in Appendix-IIIA, -IIIB and IIIC. The three types of *Acanthamoeba* isolates (Eco, Ent and Arc) also showed differences in the potential to harbour bacterial endosymbionts. The percentage of positive samples for bacterial endosymbionts out of the positive samples (30) was lower in Eco group (6/30=20%) as compared to Arc (13/30=43.3%) and Ent (11/30=36.7%). Eco also had the lowest percentage of positive samples (6/84=7.1%) out of the total samples of the same group compared to Arc (13/83=15.7%) and Ent (11/85=12.9%) (Figure 2.35). All the sequences were 98-100% identical to the Genbank database sequences.

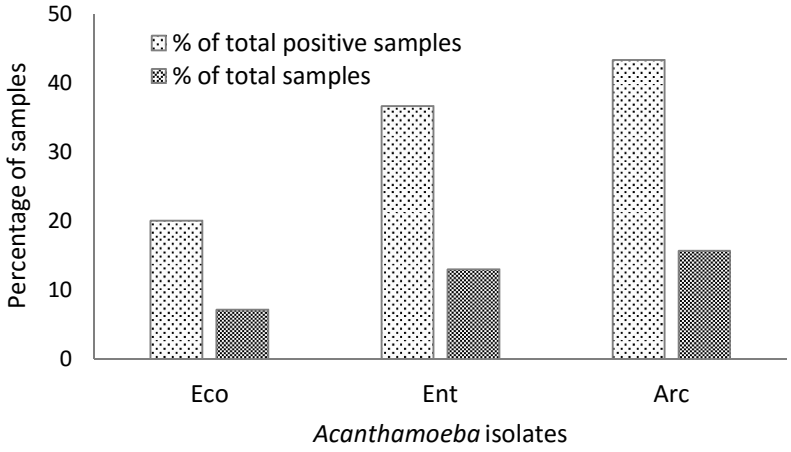


Figure 2.35: Percent distribution of the *Acanthamoeba* isolates that were detected positive for BEs. The lighter bars show percentage of isolates found positive out of the total number of isolates found positive for BEs (30). The darker bars on the other hand show percentage of isolates found positive (6, 11 and 13 respectively) out of total number of isolates for either of Eco, Ent and Arc isolates.

2.3.3.5.1. BEs of Eco isolates

Among the Eco isolates, BEs were detected only in T type T4. The subgrouping analysis revealed that these T4 isolates were T4-B (4/6=66.7%) and T4-N (2/6=33.3%). Further analaysis of BE containing T4 types down to subgrouping level indicated that these were T4-23 (4/6=66.7%) and T4-36 (2/6=33.3%) subtypes (Figure 2.36).

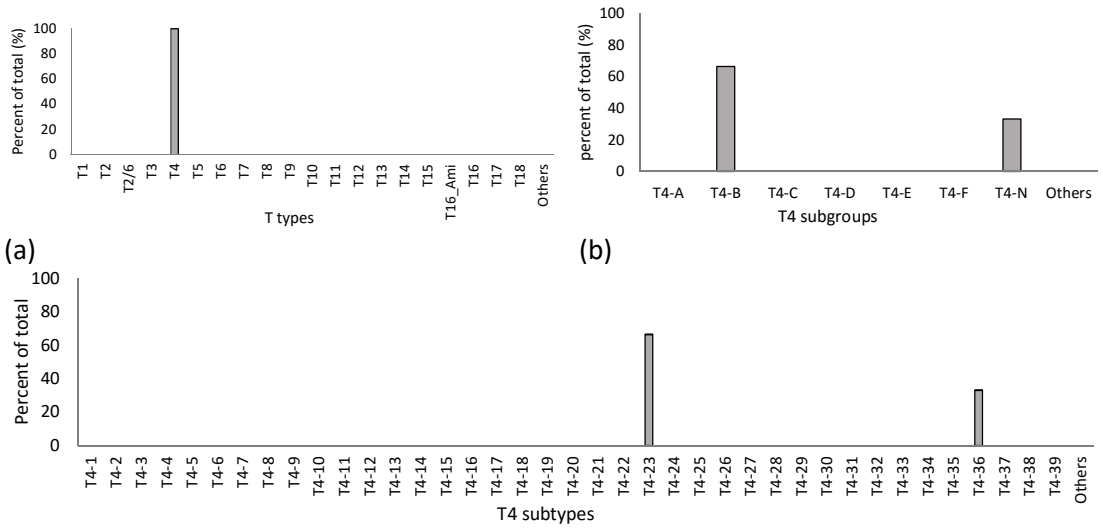


Figure 2.36: Detailed account of the BEs detected in genotypes (T types, subgroups and subtypes) of *Acanthamoeba* isolated on *E. coli*. (a) T types harbouring BEs. Only T4 isolates were found positive. (b) T4 subgroups harbouring BEs. Only T4-B and T4-N were found to contain BEs. (c) T4 subtypes harbouring BEs. Only T4-23 and T4-36 were found positive.

2.3.3.5.2. BEs of Ent isolates

Like Eco isolates, all of the Ent isolates that were detected positive for the presence of BEs beloned to T type T4. The subgroups of these T4 isolates were found to be T4-A (4/11=36.4%), T4-B (6/11=54.5%) and T4-E (1/11=9.1%). Further analaysis down to subgrouping level indicated that these T4 types belonged to subgroups T4-12 (1/11=9.1%), T4-22 (3/11=27.3%), T4-23 (6/11=54.5%) and T4-36 (1/11=9.1%) (Figure 2.37).

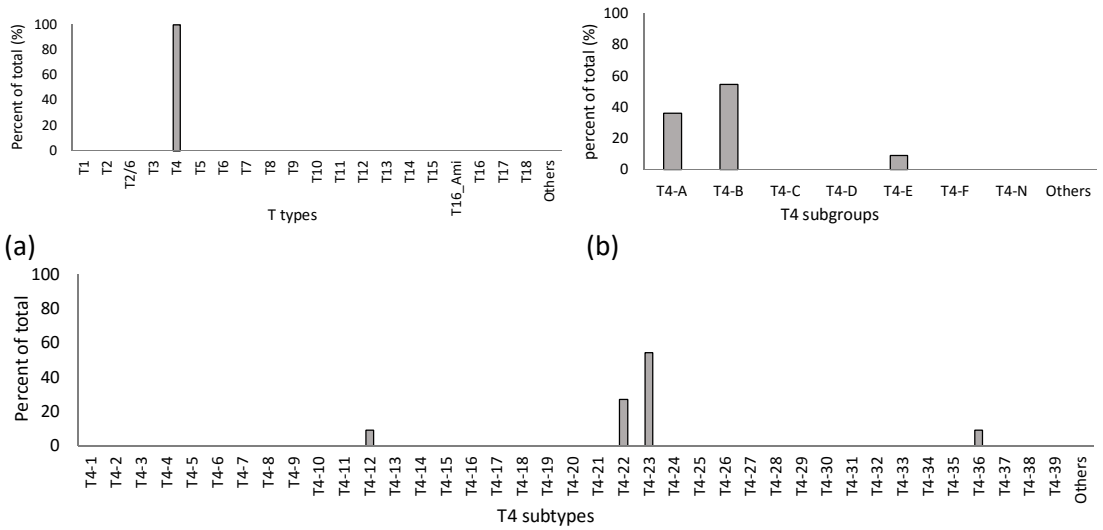


Figure 2.37: Detailed account of the BEs detected in genotypes (T types, subgroups and subtypes) of *Acanthamoeba* isolated on *Enterococcus*. (a) T types harbouring BEs. Like Eco isolates, only T4 isolates were found positive. (b) T4 subgroups harbouring BEs. Compared to Eco isolates (with T4-B and T4-N only) more T4 subgroups were found to contain BEs (T4-A, T4-B, T4-E and T4-N) in Ent isolates. (c) T4 subtypes harbouring BEs T4-12, T4-22, T4-23, T4-36 were found positive.

2.3.3.5.3. BEs of Arc isolates

Unlike Eco and Ent isolates, among Arc isolates T2 types (2/13=15.4%) were also found to be positive for BEs apart from T type T4 (11/13=84.6%). At the subgroup level the T4 isolates belonged to subgroups T4-A (2/11=18.1%), T4-B (3/11=27.3%), T4-E (3/11=27.3%) and T4-N (3/11=27.3%). At the subtype level, the T4 types belonged to subtypes T4-12 (3/11=27.3%), T4-23 (3/11=27.3%), T4-35 (2/11=18.1%) while 3/11=27.3% belonged to subtypes that were not recognized to match any of previously claimed subtypes of T4 (Figure 2.38).

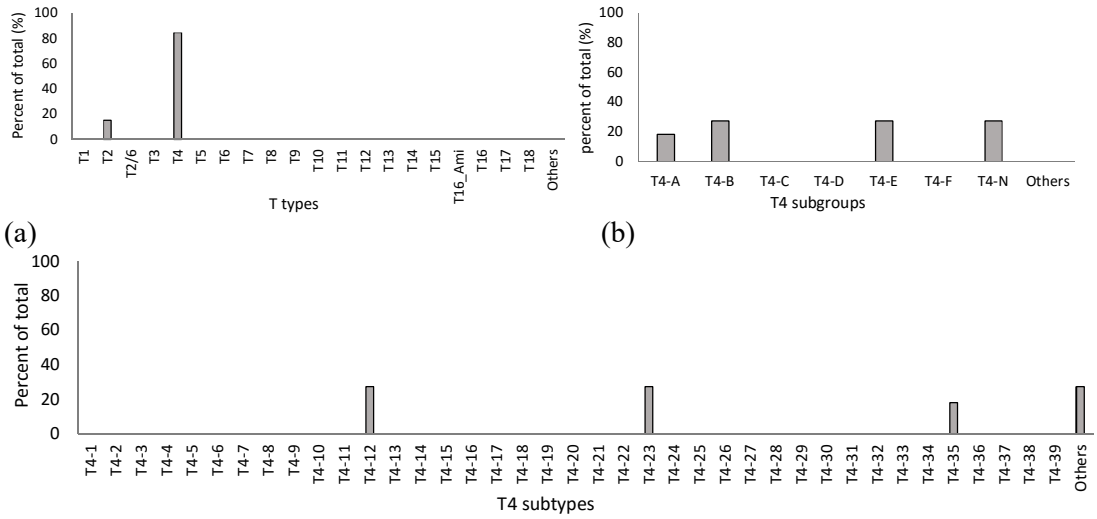


Figure 2.38: Detailed account of the BEs detected in genotypes (T types, subgroups and subtypes) of *Acanthamoeba* isolated on *Arcobacter*. (a) T types harbouring BEs. Unlike Eco and Ent isolates, apart from T4, T2 was also found positive. (b) T4 subgroups harbouring BEs. Compared to Eco isolates (with T4-B and T4-N only) and Ent isolates (with T4-A, T4-B and T4-E only), more T4 subgroups were found to contain BEs (T4-A, T4-B, T4-E and T4-N) in Ent isolates. (c) T4 subtypes harbouring BEs. T4-12, T4-23, T4-35 and other subtypes that did not belong to any of the claimed subtypes were also found positive.

Regarding the type of BEs recovered, overall, *Candidatus* Procabacter was the most abundantly detected BEs in all the *Acanthamoeba* isolates followed by uncultured bacteria and *Massilia* sp. Majority of *Candidatus* Procabacter (10/30=33.3%) out of the total endosymbionts (30) were detected in Arc isolates followed by Ent (8/30=26.7%) and Eco (6/30=20%) while *Massilia* sp. was only detected in Arc *Acanthamoeba* isolates (Figures 2.39).

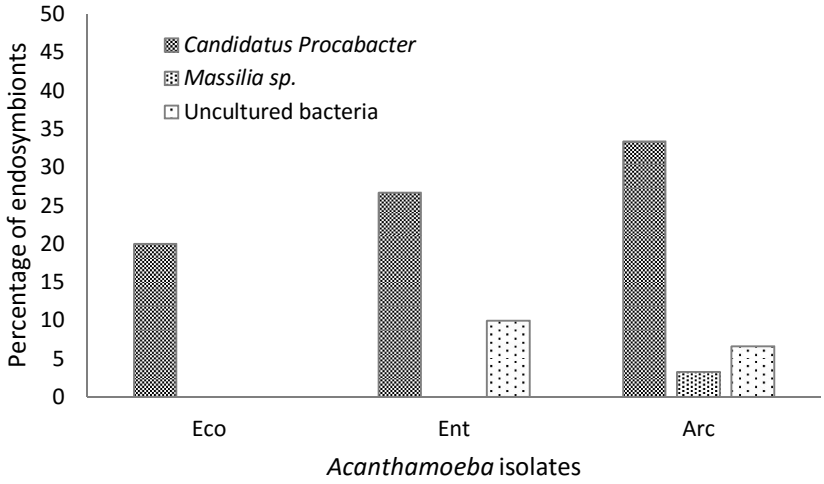


Figure 2.39: Percent distribution of bacterial endosymbionts detected in Eco, Ent and Arc *Acanthamoeba* isolates. *Candidatus* Procabacter was the most abundant bacterial endosymbionts found.

The results for the occurrence of BEs among the Eco, Ent and Arc isolates indicates likelihood of more *Acanthamoeba* types be isolated on non-*E. coli* bacteria (*Arcobacter* and *Enterococcus*) harbouring BEs because of poorer ability of *E. coli* to isolate such genotypes.

Overall, the findings of this chapter firstly indicate that the isolation modifications of modified method can significantly reduce the total time for isolation of *Acanthamoeba* from soil. Secondly, the impact of bacteria on recovery of *Acanthamoeba* genotypes is clearly evident. A single type of bacteria cannot successfully isolate all the genotypes of *Acanthamoeba* present in a sample. This may seriously affect the prevalence and diagnostic studies and ultimately affect the control & treatment strategies. It is, therefore, likely to use a combination of different types of bacteria for a more realistic picture of the various genotypes of *Acanthamoeba* present in a sample.

2.4. Discussion

This chapter deals with exploring the first objective as mentioned in Chapter 1 (section 1.1.11.1.) i.e. impact of bacteria on *Acanthamoeba*. More specifically it focuses on the role of a single type of bacteria on recovery of various genotypes present in the sample during isolation of *Acanthamoeba*. The specific question addressed was "can a single bacterium type, like the traditionally used *E. coli*, recover all the genotypes present in a sample (soil)?". However, because of the variations and long duration of isolation process, the procedure for isolation of *Acanthamoeba* from soil was first optimized.

2.4.1. Optimization of *Acanthamoeba* isolation procedures

In the current study, the optimization of the procedures for the isolation of *Acanthamoeba* from soil was important, firstly, to overcome the delaying hurdles encountered during the isolation and adaptation process to enable the processing of a wide number of samples in a reasonably short time. Secondly, it was important to make sure that the isolation process works equally well, not only for *E. coli* but also for *Enterococcus* and *Arcobacter* as these bacteria have not been used for isolation of *Acanthamoeba* before.

The isolation of *Acanthamoeba* involves two main steps i.e. monoxenic isolation using bacterial carpet as food and axenization to adapt the isolated amoebae to axenic mode of culturing. Both the isolation and axenization are time consuming. The challenges of overcoming contamination and slow axenization process make the method further laborious (Zanella *et al*., 2012). A variety of fungal contamination from environmental samples like soil is quite expected and in some cases it may be difficult to avoid fungal contamination even after rigorous treatment like HCl. Therefore, various modifications were used in the procedure to overcome the challenges and to reduce the time to minimum.

2.4.1.1. Optimization of monoxenic isolation

To keep the fungal contamination to low level and to increase the number of amoebae, different strategies were used. Firstly, small amount of soil was used (5-10 mg) as

compared to larger amounts used by most other workers, for example, 1g (Chung *et al*., 1996; Kong *et al*., 1995), 10 g (Todd *et al*., 2014), 100-200 g (Rahdar *et al*., 2012). Secondly, before transferring samples to agar plates with bacteria, soil samples were kept in saline with washed bacteria to be used by amoebae to increase their number. Thirdly, these soil samples in saline were serially diluted (two-fold) to spread out the density of any fungal contamination. Furthermore, the soil sample in saline was spread onto the surface of agar plate with overlaid bacteria to further decrease the chances of fungal contamination. This worked very well and at least one of the dilutions in each case had contamination free amoebal growth. Some workers have used micromanipulator to pick individual cysts and transfer them to the new plate (Ertabaklar *et al*., 2007; Tsvetkova *et al*., 2004; Walochnik *et al*., 2000; Zanella *et al*., 2012). However, this method is not only time consuming but also require special equipment and skills to carry out the isolation of individual cysts.

In the current study, an attempt was made to achieve the uniformity of the procedures so that variations can be kept down to the minimum for more reliable results (section 2.3.2.1.). All the three types of bacteria (*E. coli*, *Enterococcus* and *Arcobacter*) used for isolation of *Acanthamoeba* from soil were found to be killed completely at 80ºC for 10 min, however, the time required to for bacterial suspension to reach 80ºC from room temperature (heating phase) in an oven at 80ºC was much lower with intermittent shaking (50 min) as compared to 200 min when left undisturbed. Therefore, 50+10=60 min was used to kill the bacteria effectively. For the preparation of agar plates with bacteria overlaid, the best OD of bacterial suspension was found to be 1.0 which was most effective to make a good and uniform layer of bacteria. Although minor but these parameters were studied in fine detail to ensure consistency of procedures.

2.4.1.2. Optimization of axenization of *Acanthamoeba* isolates

2.4.1.2.1. Optimization of growth media for *Acanthamoeba* isolates

In the current study, AX2 media was used as a basal media and the effect of addition of various substances to this media was studied in detail by monitoring the growth of *Acanthamoeba* Neff in these media separately in addition to LB and VD media (section 2.3.2.2.). The various components added included additional yeast extract (1% instead

of 0.75% in AX2=AX2+), yeast conditioned media (yeast cultured in AX2), gentamicin-penicillin-streptomycin (GPS) at 1/10th the normal dose i.e. 100 µg/mL, 100 U/mL and 100 µg/mL respectively (Schuster, 2002) and growth promoters/plant fertilizer (GP) at various concentration levels. Among these modified media, better growth was observed in case of AX2+ (AX2 with additional yeast extract i.e. 1%) and when 0.01% of plant growth promoters or yeast-grown AX2 media were used. However, long term effects on growth were observed with AX2+, therefore, this was selected for routine use. Moreover, low doses of gentamicin-penicillin-streptomycin (1/10th normal dose) not only had no adverse effect on growth of *Acanthamoeba* but also prevented bacterial contamination of the cultures effectively. Gentamicin-penicillin-streptomycin have been used for *Acanthamoeba* for a long time (Akya *et al*., 2009; Maghsood *et al*., 2005; Schuster, 2002). However, effect of 1/10th normal doses has not been studied which was found not only to be good enough for controlling bacterial contamination at this low dose but rather had positive effect on growth as well. This can be used for routine cultures of *Acanthamoeba* saving additional dose as well.

The PYG (containing peptone, yeast extract and glucose) is a basic medium widely used for the axenic culturing of *Acanthamoeba*. The original medium used by Neff consisted of 0.75% , 0.75% and 1.5% of these components respectively (Neff, 1957). However, various proportions of these basic components have been used for axenization of *Acanthamoeba* cultures widely (Kong *et al*., 1995; Lorenzo-Morales *et al*., 2005; Reyes-Batlle *et al*., 2014; Visvesvara *et al*., 1975). Many modifications and other media have also been tried. At many instances other media had to be used. Tsvetkova *et al*., (2004) used two liquid media for axenization of *Acanthamoeba* i.e. PPG consisting of proteose-peptone glucose (1.5%, 1.8% in Page's amoeba saline) and yeast extract-PAS medium (YAS) which is 0.1 g yeast extract in 1,000 ml Page's amoeba saline). Similarly Rezaeian *et al*., (2008) used TYI-S-33 medium consisting of 0.1 g of potassium phosphate dibasic, 0.06 g of potassium phosphate monobasic, 0.2 g of sodium chloride, 0.2 g of casein digest peptone, 2 g of yeast extract, 1 g of glucose, 0.1 g of L-cysteine hydrochloride, 0.1 g of ascorbic acid, and 0.0023 mL of ferric ammonium citrate. The cultures were then grown in PYG media. Kong *et al*.,

(1995) used PYG with added L-cystein. Rahdar *et al*., (2012) attempted PYG in the first instance but amoebae were not adopted to this medium, therefore, TYI-S-33 medium was used.

Defined media for specific species of *Acanthamoeba* have also been used. A modification of PYG consisted to 18 added amino acids and vitamin B12 with acetate as carbon source was used for Neff strain (Adam, 1959). Later while working with a number of other strains including Neff, Band (1961) realized that biotin was also needed for. Similarly, another medium for *Acanthamoeba rhysodes* was formulated consisting of glucose/sodium acetate (carbon source) along with seven amino acids, biotin, vitamin B12 and thiamine (Band, 1962). Another recipe used for various strains of *Acanthamoeba* contained methionine, arginine, isoleucine, leucine and valine in basal medium (Adam and Blewett, 1967). They found variations in the use of sucrose, melibiose, mannitol, and raffinose as carbon energy sources. Variable growth rates were observed with these media usually with delayed generation times upto 40-60 h. A better generation time of 13-16 h was reported for medium DGM-21A which was quite complex. It contained 21 different amino acids, seven vitamins, seven salts, seven trace element sources, glucose, Na acetate and ethanol (Byers *et al*., 1980). De Jonckheere (1980) used another medium containing yeast extract, glucose and casein for *Acanthamoeba* isolates, however, it could not work well for all the isolates as some of them still required a richer medium like PYG with added vitamins and serum but still the growth was not adequate (Schuster and Visvesvara, 1998).

In the current study the attempts were made to modify the culture media without involving complex changes. The tested media were evaluated for extended periods of time so that the amoebal growth can be sustained for longer without causing excessive encystation and the medium which is equally well for all the three types of isolates (Eco, Ent and Arc). These goals were successfully met.

2.4.1.2.2. Improving adaptation of *Acanthamoeba* to axenic culturing

For improving the adaptation and proliferation of *Acanthamoeba* isolates towards the axenic mode of culturing, addition of dead bacteria gave encouraging results (section

2.3.2.3.). This has not been tried before although Zanella *et al*., (2012) used dead bacteria in saline but this was only to improve isolation but not axenization. Shifting of *Acanthamoeba* from bacteria-eating culture to non-bacteria axenic culture usually results in rounding off and encysting of *Acanthamoeba* cells due to sudden change of environment. The addition of dead bacteria on AX2 agar plates and later in AX2 media was aimed at help *Acanthamoeba* cells in transition and significant reduced the time for isolation as compared with the traditional method.

Overall, the optimized method, using all these modifications, reduced the chances of contamination and also reduced the net time for isolation of *Acanthamoeba* from soil samples, not only in case of *E. coli* but also for *Enterococcus* and *A. butzleri*. This was evident by the comparison of the traditional method with the optimized method for isolation of *Acanthamoeba* from soil using 30 soil samples isolated on *E. coli*, *Enterococcus* and *Arcobacter* (10 each). With the optimized method the net time (in days) for isolation of *Acanthamoeba* was significantly lesser than traditional method (13.7±0.25 vs 26.3 ±1.67) (Table 2.4).

These findings are not only interesting but also important with regard to overcoming the problems encountered during the isolation of *Acanthamoeba* from soil making the procedure time consuming. Simple modifications were used which are easy to be followed. The findings of the study clearly suggest that taken together, the modifications implemented (amoebae enrichment, dilution/spreading technique, addition of dead bacteria during transition phase and use of AX2+ media) have an impact on speeding up the overall isolation process. Furthermore, this method can not only be used for *E. coli* but also for other bacteria not least including *Enterococcus* and *Arcobacter*. It is hoped that the improved method can also be used for isolation of *Acanthamoeba* from other samples sources like water, waste materials and tissues. Moreover, low doses of antibiotics combination (gentamicin, penicillin, streptomycin at 100 µg/mL, 100 U/mL and 100 µg/mL respectively) may be used for routine culturing of *Acanthamoeba* as it not only saves antibiotic but also doesn't seem to cause encystation, rather better trophozoite growth. Although comparable growth of

Acanthamoeba was also observed with the addition of plant growth promoter, however, it may be of interest to further investigate the individual components.

2.4.2. Role of bacteria in selection of *Acanthamoeba* genotypes from environmental samples

Use of *E. coli* as monoxenic source of food is currently a standard procedure for the isolation of *Acanthamoeba* (Schmitz-Esser *et al*., 2008) although other organisms including *Enterobacter* and *Saccharomyces cerevisiae* have rarely been used as well (Heinz *et al*., 2007; Schmitz-Esser *et al*., 2008). Over the decades, by using *E. coli* as food a particular set of T types of *Acanthamoeba* has been recognized (T1-T19) (Astorga *et al*., 2011; Corsaro and Venditti, 2010; Di Cave *et al*., 2009; Edagawa *et al*., 2009; Ertabaklar *et al*., 2007; Horn *et al*., 1999; Huang and Hsu, 2010; Łanocha *et al*., 2009; Liang *et al*., 2010; Liu *et al*., 2006; Magnet *et al*., 2012; Qvarnstrom *et al*., 2013; Walochnik *et al*., 2000a; Yera *et al*., 2007). However, it is not clear that if other type of bacteria are used instead of *E. coli*, then the same genotypes/subtypes will be recovered or the choice of bacteria used can affect the outcome. This important question has never been addressed. For this purpose three different type of bacteria including *E. coli*, *Enterococcus* and *Arcobacter* were used as selection media (food) for the isolation of *Acanthamoeba* from the soil samples. Each sample was processed on either of *E. coli*, *Enterococcus* or *A. butzleri* while ASA.S1 region of 18S rRNA was used for sequencing.

In the current study *E. coli* were used as control as these are mainly used for this purpose. *Enterococcus* and *Arcobacter* were used as these are normally present in the environment and since the samples were also environmental (soil) so these might reflect better natural scenario. Furthermore, the choice of the two bacteria also provided a comparison of *Acanthamoeba* isolation of Gram+ve (*Enterococcus*) and Gram-ve (*Arcobacter*) bacteria.

Some basic studies dealing with amoebae's ability to feed upon different bacteria were undertaken by Singh (1941), Singh (1942) and Singh (1946). More elaborate studies were, however, carried out by Weekers *et al*., (1993) to find out amoebal preference

of different types of bacteria as food. The non-pigmented bacteria *E. coli* K-12 and *Klebsiella aerogenes* were found to serve as better food for *Acanthamoeba*. Barely any growth was observed in pigmented bacteria (*Chromatium vinosum* and *Serratia marcescens*) while low to moderate growth was observed in indigenous soil bacteria (*A. tumefaciens, A. simplex, B. megaterium, B. subtilis, M. luteus* and *P. fluorescens*). Schuster (1993) also reported a preferential feeding response for different types of bacteria. By studying various bacteria, these were divided into different groups including readily eaten (*Bacillus cereus* and *Enterobacter cloacae*), moderately consumed (*Staphylococcus aureus*), poorly consumed (*Serratia marcescens*), and inedible (*Pseudomonas aeruginosa* and *Chromobacter violaceum*).

Khan (2009) reported that *Acanthamoeba* preferentially feed upon Gram-ve bacteria but not Gram+ve bacteria. This was supported by findings of this study as well as more diverse genotypes/subtypes were recovered in case of Arc (Gram-ve) isolates rather than Ent (Gram+ve) isolates of *Acanthamoeba* as discussed below. However, the detailed effect of choice of bacteria as food on selection of *Acanthamoeba* genotypes and their various characteristics has never been investigated.

2.4.2.1. Comparison of Eco, Ent and Arc genotypes

A very good recovery of *Acanthamoeba* from soil samples was achieved in the current study. Out of the 102 samples processed, each on *E. coli*, *Enterococcus* and *Arcobacter* (102×3=306) 12 of the samples (total 12×3=36) were negative for *Acanthamoeba* because they contained no *Acanthamoeba* as determined by genus-specific PCR (Appendices IA, IB & IC) thus a recovery of 90/102 (88.2%) which is much higher than that of Lorenzo-Morales *et al*., (2005) who recovered *Acanthamoeba* from only 43/114 (37.7%) soil samples.

Overall, Eco isolates showed the least diversity while the Arc isolates had the most divergent sequences. This was evident as most of the Eco isolates sequences had near perfect (upto 100%) similarity with the already reported sequences *viz* 52.8% compared to 21.7% for Arc. All the Eco isolates were in narrow similarity range of 98-100% while 2.3% of Ent and 1.2% of Arc isolates were at even further down the 98%

similarity level. Apart from that, some of the sequences found in case of Arc isolates were quite divergent compared to the reference sequences as discussed below. This indicates that more subtypes or subgroups exist which can be isolated using bacteria other than *E. coli*.

2.4.2.1.1. Sequence diversity

The T types identified in Eco isolates included T2 (6.4%), T4 (88.2%), T11 (2.1%) and T13 (3.2%) (Figure 2.19) in comparison to Ent isolates where only T4 (95.1%) and T16 (3.6%) were identified (Figure 2.23), while on the other hand, among the Arc isolates the T types T2 (14.1%), T2/6 (2.3%), T4 (76.4%), T13 (2.3%) and T16 (2.3%) were identified and one of the isolates was neither a full match for T13 or T16 but an intermediate of T13 and T16 (Figure 2.28).

In a recent study of entire databank of *Acanthamoeba* sequences by Fuerst (2014), based on partial 18S rRNA gene sequence the T types reported are in the order of T4 (71.6%) > T5 (6.1%) > T3 (5.8%) > T6 (4.1%) > T15 (2.8%) > T2 (1.9%) > T11 (1.6%) > T19 (1.0%) > T1 (0.8%)=T13 (0.8%) > T12 (0.5%)=T17 (0.5%) > T7 (0.3%)=T10 (0.3%) > T9 (0.2%) > T8 (0.1%) > T16 (0.05%) > T14 (0%)=T18 (0%), while the T types T14 and T18 on the basis of full length sequence are 0.6% and 0.3% respectively. Although in the current study all the Types were not recovered, however, interestingly in all the three types of isolates (Eco, Ent and Arc), the order of frequency of Types recovered (Eco T4 (89.2%) > T2 (6.0%) > T11 (2.4%) = T13 (2.4%); Ent T4 (95.1%) > T16 (3.7%) > others (1.2%); Arc T4 (78.6%) > T2 (16.7%) > T13 (2.4%) others (2.4%)) was the same as that of overall sequences deposited in databank.

2.4.2.1.2. T4 sequences

The diversity of T4 sequences was compared for the three types of isolates. Overall the Ent T4 isolates had highest divergence (upto 4%) from the references sequences (Figure 2.24) as compared to 0-2% divergence of Eco (Figure 2.20) and Arc isolates (Figure 2.29). It is interesting that among all the three types of *Acanthamoeba* isolates (Eco, Ent and Arc) T4 was the major T types recovered (Figures 2.19, 2.23, 2.28). This is in consistence with the finding of Maciver *et al*., (2013) and Fuerst (2014).

2.4.2.1.3. T4 Subgroups

In all the three cases for *Acanthamoeba* isolates (Eco, Ent, Arc), the isolates belonged to either of the six out of seven subgroups of T4 (T4-A, T4-B, T4-C, T4-D, T4-E, T4-F and T4-N), however, none of the isolates belonged to T4-F. There was a pronounced difference in the abundance of T4 subgroups recovered in Eco, Ent and Arc isolates with T4-A being the major type in Eco and Ent isolates while T4-E was the most abundant subgroup of Arc isolates (Figure 2.20, 2.24, 2.29).

The order of frequency of T4 subgroups among the overall sequences submitted to databanks is T4-A (47.8%) > T4-B (19.4%) > T4-D (13.3%) > T4-E (8.6%) > T4-C (6.6%) > T4-N (2.6%) > T4-F (1.7%) (Fuerst, 2014). This order was closely similar to that of Eco isolates (T4-A (54.1%) > T4-B (16.2%), T4-N (10.8%), T4-E (9.5%), T4-D (8.1%) and T4-C (1.3%)) and to some extent in Ent isolates (T4-A (47.0%) >T4-E (12.3%) > T4-C (11.1%)=T4-D (11.1%) = T4-N (11.1%) > T4-B (7.4%)) but quite varied in Arc isolates (T4-E (34.8%) > T4-A (28.8%) > T4-B (19.7%) > T4-N (16.7%)). This close similarity of Eco isolates to that of databank isolates is probably due to the fact that *E. coli* are widely used for the isolation of *Acanthamoeba* while greater variation was seen in case of *Arcobacter* isolates which have never been used for isolation of *Acanthamoeba*. This indicates that the use of bacteria other than *E. coli* may present a different prevalence of *Acanthamoeba* genotypes.

2.4.2.1.4. T4 subtypes

Overall, out of the 38 subtypes of T4, 15 subtypes were identified while a group of subtypes in all the three cases could not be assigned to any type because of very low similarity index to the other subtypes and were categorized as “others” (Figure 2.21, 2.25, 2.30). This is not surprising as the T4 subtype system is not fully agreed upon with many of the overlapping names (T4-1 to T4-39 had to be assigned to avoid any confusion) (Abe and Kimata, 2010; Booton *et al*., 2002; Ledee *et al*., 2009; Magnet *et al*., 2013; Risler *et al*., 2013; Zhao *et al*., 2010). There are many T4 sequences in the Genbank where the T4 subtype has not been identified. The highest percentage of the low-match subtypes was found in Arc isolates. This finding was again consistent with the finding above that the most divergent sequences are found in case of Arc isolates.

Interestingly some of the T4 subtypes were unique to Ent (T4-7 and T4-27) and Arc (T4-31 and T4-34). Similarly, T4-22 was found only in Ent and Arc isolates. There was also lack of agreement between the most abundant T4 subtype among the three types of *Acanthamoeba* isolates; T4-36 being the largest subtype both in Eco and Ent isolates while Arc isolates again had difference with T4-12 being the most abundant subtype. There were marked differences among other subtypes in the three groups. These results clearly indicate that the whole variety of subtypes of *Acanthamoeba* cannot be recovered by using just one type of bacteria.

2.4.2.1.5. Other T types

Apart from the fact that overall the most divergent and diverse sequences were found among the Arc isolates, the T2/6 isolates were unique to Arc samples only while the intermediate T13/16 were found in both Arc and Ent isolates (Figure 2.28 and 2.23). Moreover, the T13 and T13/16 isolates from Arc group (Figure 2.31 and Appendix-IC) and T13/16 sequence from Ent group (Figure 2.26 and Appendix-IB) had 2-3% divergence from not only the reference T types but Genbank database sequences as well which is an important finding and there may be other hidden genotypes that may be revealed by use of bacteria other than *E. coli*. Therefore, these sequences may be new subtypes but this will require a full length sequencing of the 18S rRNA. Among all the three isolation groups, the T2 type was recovered in greatest proportion from Arc group with divergence of upto 6% from the reference sequences. T11 isolates were only recovered from Eco samples. These findings were also in consistence with the phylogenetic reconstruction analysis.

It is clear from these results that the level of divergence and diversity among the non-T4 isolates was the greatest in Arc and Ent isolates as compared to the Eco isolates while the maximum diversity and variety of T4 subgroups was found in Eco isolates.

2.4.2.2. Pathogenic potential of Eco, Ent and Arc isolates

The pathogenic characteristics (thermotolerance and osmotolerance) were not quite useful in differentiating the three isolate groups as no effect of osmotolerance or thermotolerance at 41°C could be observed (Appendix IIA, IIB, IIC). At 37°C majority

of isolates had poor growth. Although such comparison has not been made before but studies have shown variable results. Landell *et al*., (2013) studied thermotolerance of nine environmental (bromeliads) *Acanthamoeba* isolates at 42°C during 10 days and found 0/10 (0%) positive. Niyyati *et al*., (2013) observed the *Acanthamoeba* environmental cultures (soil, dust and water) from 24-72h at 42°C and found 3/10 (30%) thermotolerant respectively. Also, similar osmotolerance findings have been reported by Landell *et al*., (2013) who studied osmotolerance of nine environmental *Acanthamoeba* isolates at 1M mannitol during 10 days but found none of the nine isolates positive. On the other hand Niyyati *et al*., (2013) found 3/10 (30%) environmental cultures (soil, dust and water) osmotolerant to 1M mannitol. Lorenzo-Morales *et al*., (2005) found 54.5% of *Acanthamoeba* environmental samples osmotolerant.

Thermotolerance and osmotolerance are considered indicators of pathogenic potential. Human eye has a temperature of 34ºC while brain is at 37ºC, therefore, to cause infection *Acanthamoeba* should be able to survive these temperatures (Brown *et al*., 1982; Pumidonming *et al*., 2010; Walochnik *et al*., 2000). Inability to survive the stressful environment of mannitol and high temperature of 41°C by the isolates with only limited survival of Eco and Ent isolates at 37ºC, indicates possibly limited or no pathogenic potential of these isolates respectively. This might probably be due to environmental nature of the samples (soil).

2.4.2.3. Bacterial endosymbiontic profile of Eco, Ent and Arc isolates

The relation between the *Acanthamoeba* isolates (Eco, Ent and Arc) and the type of BEs they harbour was studied. Although this kind of work has not been done before, the presence of BEs in *Acanthamoeba* (isolated on *E. coli*) has been undertaken by different workers (Fritsche *et al*., 1993; Fritsche *et al*., 1999; Heinz *et al*., 2007; Horn *et al*., 1999; Horn and Wagner, 2004; Schmitz-Esser *et al*., 2008).

In the current study, BEs were detected in 7.1% (6/84), 12.9% (11/85) and 15.7% (13/83) of Eco, Ent and Arc isolates respectively (Figure 2.35) indicating comparatively a stronger correlation between the *Acanthamoeba* T types isolated on

Arcobacter and the BEs. Furthermore, *Candidatus* Procabacter was found to be the major BE among the *Acanthamoeba* isolates which indicates their widespread distribution. This is in consistence with the findings that the majority of BEs belong to Proteobacteria (Horn *et al*., 2002). *Candidatus* Procabacter have been reported from *Acanthamoeba* isolated from a variety of sources including freshwater, saline lake, desert, soil, lake sediment and human cornea (Heinz *et al*., 2007; Horn *et al*., 2002; Schmitz-Esser *et al*., 2008).

Among the T4 subgroups T4-B and among T4 subtypes T4-23 appear to be more prone to harbour BEs than others (Figure 2.36, 2.37, 2.38). However, with more and more positive samples a broader picture can be visualized. The recovery of majority of BEs from T4 isolates is probably related to the greater proportion of this T type and is consistent with previous studies (Fritsche *et al*., 1999; Schmitz-Esser *et al*., 2008).

Among the Eco and Ent isolates, BEs were detected only in T type T4 in contrary to Arc isolates where T2 were also found to contain BEs. Schmitz-Esser *et al*., (2008) also found BEs in six T4 and two T2 isolates. They found BEs in 8/10 (80%) *Acanthamoeba* isolates from environmental samples. *Candidatus* Procabacter was found in one of each T4 and T2 isolates other than *Candidatus* Amoebophilus, *Candidatus* Amoebophilus and *Parachlamydia* spp.

For four groups of bacteria stable associations with amoebae leading to long-term endosymbiosis have been observed including Alphaproteobacteria (Horn *et al*., 1999), the Betaproteobacteria (Horn *et al*., 2002), the Bacteroidetes (Horn *et al*., 2001), and the Chlamydiae (Horn *et al*., 2000). None of the bacteria belonging to these groups have the ability to survive outside the host and, therefore, cannot be cultured in vitro in the laboratory. This kind of relation can have clinical importance as *Acanthamoeba* might play role in protecting and then releasing the contained BEs as manifested by the fact that people with AK have been reported to have other infections as well like Herpes simplex virus (HSV), Adenovirus and *Pseudomonas* species (Rumelt *et al*., 2000).

It is clear that the test *Acanthamoeba* isolates (Ent and Arc) in this study not only differed from control isolate Eco in their T typing, subgrouping and subtyping but also the number and variety of BEs they harboured. Overall, the majority of BEs were found linked to T4-B subgroup and T4-23 subtype. T4-B accounted for ~20% of Arc isolates compared to ~7% of Ent and ~16% of Eco isolates which shows that more *Acanthamoeba* belonging to T4-B can be isolated when *Arcobacter* are used for isolation and ultimately more BEs or in other words using only *E. coli* as prey bacteria can results in a limited picture of the prevalence of BEs. There is no previous extensive study with BEs that can be used for comparison although there are studies that involved detection of BEs from environmental samples or clinical cases but with a small number of samples. Schmitz-Esser *et al*., (2008) studied eight samples out of which six were T4. Although these were not analysed down to subgroup or subtype level like other studies, however, for the sake of comparison of current work, this has been analysed to subgroup and subtype levels. One of these was T4-B (two N, one A and two D) and none of these belonged to T4-23.

The detailed knowledge about the BEs of *Acanthamoeba*, except chlamydia-related symbionts, is limited (Schmitz-Esser *et al*., 2008). Although there still remains a lot to learn about the complex phenomenon of interaction between *Acanthamoeba* and bacteria, yet this study provides convincing evidences that the choice of bacteria used for the isolation of *Acanthamoeba* may not only provide a different picture of *Acanthamoeba* genotyping system (at least at subtyping and subgrouping level if not at T types level) and the prevalence of these types in the environment but also we may see a different picture of the recovered BEs. These findings can further be consolidated with more and more samples processed with diverse types of bacteria used for isolation.

2.5. Conclusion

Overall, the findings of the post optimization (i.e. role of bacteria other than *E. coli* in unmasking genotype/subtypes of *Acanthamoeba*) indicate a very important observations. It is clear from the results that full set of T types/subgroups/subtypes of *Acanthamoeba* from environment (soil) cannot be recover by using just one type of bacteria (*E. coli*). This was revealed by the recovery of different sets of T types, subgroups and subtypes of *Acanthamoeba* when the soil samples were processed in triplicate each on *E. coli*, *Enterococcus* and *Arcobacter*. Isolation of some genotypes differentially (e.g. T2/6 only in Arc isolates and T13/16 only in Ent and Arc isolates) and others' preferentially (more T2 types in Arc isolates) requires a careful consideration. Furthermore, the Arc isolates of *Acanthamoeba* were not only different in their sequence diversity but also in their morphology and bacterial endosymbiotic profile.

Recovery of a different set of genotypes in Arc and Ent isolates is a significant finding as this may alter the geographical distribution studies for *Acanthamoeba* as possibly more and different genotypes can be recovered in this case. Furthermore, this can also have a serious effect on the determination of the exact genotype(s) associated with the clinical cases like GAE and AK and this will push the limitation further down to designing appropriate treatment and prevention strategies.

Although this was a preliminary study the message is clear. Based upon the findings of this study, the conclusions can be summarized as follows:

2.5.1. The isolation of *Acanthamoeba* from soil can be improved with overall reduced time and lesser contamination by introducing the modifications (increasing the number of amoebae by addition of bacteria, dilutions before adding onto agar plate with bacteria, use of dead bacteria while transition from monoxenic to axenic culture).

2.5.2. The type of bacteria used for isolation of *Acanthamoeba* from soil affect the T types of *Acanthamoeba* recovered.

2.5.3. *Acanthamoeba* isolated on *Arcobacter* and to lesser extent those on *Enterococcus* have greater diversity of sequences than those isolated on *E. coli*.

2.5.4. *E. coli* isolates of *Acanthamoeba* have greater variety of T4 subtypes than *Enterococcus* and *Arcobacter* isolates.

2.5.5. More/new genotypes/subgroups/subtypes may possibly be found if a combination of different bacteria is used which can give a better picture of the distribution of various T types of *Acanthamoeba* in the environment.

2.5.6. *Acanthamoeba* isolated on *Arcobacter* seem to have greater chances of the harbouring BEs inside them. *Candidatus* Procabacter appears to be the most abundant BEs in *Acanthamoeba* from soil samples in UK.

2.6. Further investigations

Further full-length sequence analysis of T13/16 intermediate Ent and Arc isolates is needed to be carried out to confirm whether these are new T types or subgroups. Although this study clearly indicates the effect of bacteria on the recovery and diversity of *Acanthamoeba* genotypes, this study can be expanded to large scale with a broader range of different types of bacteria and a broader range of samples from a variety of environmental matrices and clinical samples as well. Moreover, full-length 18S rRNA sequence analyses will be required to confirm the findings. A well-defined protocol should be produced that recommends use of a combination of different types of bacteria or use of different bacteria separately for the isolation of whole variety of genotypes present in a sample based upon the findings of this study. Although these suggestions will require resources and time but this may help reveal further genotypes/subtypes and thus enable to understand better the phenomenon.

The other aspects of *Arcobacter-Acanthamoeba* relationship have been further explored in detail in the next chapter (Chapter 3).

Chapter 3

Impact of *Acanthamoeba* on bacteria-I: Interaction of *Acanthamoeba* with *Arcobacter butzleri*

Abstract

This chapter deals with investigating influence of *Acanthamoeba* on bacteria taking first of the two examples of emerging human bacterial pathogens-*Arcobacter butzleri*. These bacteria are also abundantly present in the environment where they have opportunity to interact with free living protozoans like *Acanthamoeba*. The role of *Acanthamoeba* in acting as environmental reservoirs of bacteria and in enhancing the virulence of bacteria as a part of the process of adaptation for human infections is well documented for many bacteria. However, the interaction of *A. butzleri* with *Acanthamoeba* and the possible implications of this interaction on human health has not been investigated. *A. butzleri* appeared to be easily located and approached by *Acanthamoeba* as demonstrated by strong chemotactic attraction for these bacteria. Furthermore, they attached to the amoebae instantly forming a multi-layered "cap" on the cell surface. Entry into the cell was also quick and appeared to depend upon monosaccharide sugar receptors on the cell surface of amoeba. Phagocytosis was a complex process and required various cellular signalling pathways to be accomplished as demonstrated by the use of specific phagocytosis inhibitors including actin polymerization inhibitor (cytochalasin D), PI3K inhibitor (wortmannin) and tyrosine protein phosphatase inhibitor (sodium orthovanadate). Survival in amoeba was found to be related to the requirement for defying the acidification processes as shown by use of a weak base (ammonium chloride), H^+/Na^+ ionophore (monensin), inhibitor of lysosome-phagosome fusion (suramin) and inhibitor for v-ATPases (bafilomycin A). *A. butzleri* showed ability to infect *Acanthamoeba* cells, with limited proliferation, cell lysis and weak cell-to-cell spread. Although majority of internalized bacteria were killed within 24h, bacteria could survive for up to 14 days. Intracellular survival of *A. butzleri* in *Acanthamoeba* enhanced their pathogenic potential towards amoebae (increased internalization) and demonstrated longer survival up to 42 days, indicating a vital role of *Acanthamoeba* in altering pathogenic potential of these bacteria. Role of bacterial two-component system in sensing environmental changes (presence of nicotinic acid) and thus modifying their morphology and pathogenic potential was confirmed for *A. butzleri*; additionally, together with intracellular survival in amoebae, the pathogenicity of these emerging human pathogens may increase many fold, posing a health threat. Co-culture studies indicated a definite advantage for *A. butzleri* in terms of enhanced growth, however, amoebae were negatively impacted as a result of this mutual interaction and *A. butzleri* didn't have an impact on inducing excystation. Based on the findings of this study it appears logical that *Acanthamoeba* serve as the environmental hosts for the emerging human pathogen-*A. butzleri*, which is worrying and calls for consideration of this important factor in designing control and treatment strategies for this pathogen.

3.1. Introduction

3.1.1. Interaction between *Acanthamoeba* and bacteria

Acanthamoeba being free-living amoebae (FLA) in the environment serve as a common host for environmental bacteria. Presence of both *Acanthamoeba* and bacteria in the same environment provides opportunity to interact with each other. The nature of this interaction is diverse and may vary from a typical predator-prey (where bacteria serve as food) to symbiotic (allowing bacteria to reside intracellularly) to pathogenic (whereby bacteria can not only resist hostile cellular environment but also they can proliferate and destroy *Acanthamoeba* cell, thus disseminating to other places) (Greub and Raoult, 2004; Marciano-Cabral, 2004; Tezcan-Merdol *et al*., 2004; Weekers *et al*., 1993). This was discussed in detail in Chapter 1 (1.1.9.2.3.).

Legionella's interaction with *Acanthamoeba* has been investigated in detail. This important relation has helped understand the significant role of amoebae as reservoirs and training grounds for bacteria. Intra-amoebal survival is required by *L. pneumophila* to cause human infections (Rowbotham, 1986). *L. pneumophila* use amobae as natural reservoirs and breeding grounds for their growth and survival in the environment (Rowbotham, 1986). Rupture of infected cells results in dissemination of bacteria which in the appropriate setting will act as source of infection for humans (Gao and Kwaik, 2000). Bacteria are even capable of surviving in the amoebal cysts (Kilvington and Price, 1990) which provides further protection from chemical management agents, such as chlorine (King *et al*., 1988). Therefore, *Acanthamoeba-Legionella* form an excellent model to study the dynamics of interaction between bacteria and non-mammalian cells.

Mycobacterium interaction with *Acanthamoeba* has unveiled very important aspects of bacteria-amoeba interactions. *Mycobacterium* sp. have been shown to survive in *Acanthamoeba* including *M. marinum*, *M. fortutum* and *M. avium* (Cirillo *et al*., 1997). Intracellular survival in *Acanthamoeba* was linked to enhanced virulence, decreased sensitivity to antibiotics, and better survival in mice. The significance of intracellular

survival in *Acanthamoeba* on development of pathogenic traits was clearly demonstrated in the form of increased resistance to antibiotics as a result of intra-amoebal survival of *M. avium* but not in case of intra-macrophagal survival (Miltner and Bermudez, 2000).

Campylobacter were shown to serve as a non-vertebrate reservoir for *C. jejuni* in the environment. Various strains of *C. jejuni* were found to infect *A. polyphaga* and survived in co-cultures for extended periods of time. The infecting cells were found to contain motile *C. jejuni* bacteria. The interaction also seemed to be helpful in resuscitation of bacterial cultures that were previously negative in culturability tests (Axelsson-Olsson *et al*., 2005). The uptake and intracellular trafficking of viable and heat killed bacterial cells of the *C. jejuni* in *A. polyphaga* were studied by Olofsson *et al*., (2013). The viable bacteria were found to be associated with fairly high population of amoebae as compared to dead bacteria. Bacterial viability also appreared to play an important role in internalization, number of internalized bacteria and intracellular localization of bacteria suggesting that uptake and intracellular survival of *C. jejuni* in *Acanthamoeba* is induced by these bacteria.

Acanthamoeba have been recognized to act as environmental reservoirs for a number of other pathogenic bacteria including *Salmonella typhimurium, Chlamydia pneumonia*, *Taylorella equigenitalis, Taylorella asinigenitalis* and *Burkholderia cepacia* (Allombert *et al*., 2014; Ben Salah and Drancourt, 2010; Marolda *et al*., 1999; Molmeret *et al*., 2005; Tezcan-Merdol *et al*., 2004). The relationship between amoebae and bacteria is, no doubt, crucial in an evolutionary prospect because of the possible role of amoebae in acting as training grounds for bacteria and exerting selective pressure for survival; the skills developed through this interaction are believed to have conferred capability to act as pathogens for animals and humans (Greub and Raoult, 2004; Harb *et al*., 2000).

There is no consistency in the mode of interaction between *Acanthamoeba* and bacteria as the eventual mechanisms involved depend on different factors. For example, the methods of escaping ingestion vary widely depending upon bacterial size, virulence

factors and toxin production (Adiba *et al*., 2010; Jezbera *et al*., 2006; Kinner *et al*., 1998; Matz *et al*., 2004). Similarly, all ingested bacteria may not necessarily be digested as some bacteria can evade the killing mechanisms of *Acanthamoeba* and even multiply in the cell, like *L. pneumophila* (Molmeret *et al*., 2005). Therefore, in the environment, *Acanthamoeba* are believed to support bacteria including pathogenic bacteria in their survival, transmission and pathogenicity.

Arcobacter are emerging food borne pathogens (Cardoen *et al*., 2009; Collado and Figueras, 2011). They are of great importance because of their close relation with *Campylobacter* (Ferreira *et al*., 2015) whereas campylobacteriosis (causing diarrheal disorders and bacteraemia in humans) is the most frequently reported zoonosis in industrialized countries (FSA, 2013). *Arcobacter* form a diverse group of bacteria comprising 18 different species (Ferreira *et al*., 2015). Among these, *Arcobacter butzleri* have recently received great attention and have been regarded as potential emerging zoonotic pathogens (Cardoen *et al*., 2009; Collado and Figueras, 2011). They are responsible for enteritis, colitis, septicaemia and bacteremia (Arguello *et al*., 2015; Lau *et al*., 2002) and have also been isolated from abortion and enteritis cases from livestock (Wesley and Miller, 2010). *A. butzleri* are excreted by their hosts and are thus frequently found in the environmental matrices (Collado and Figueras, 2011). These have been discussed in detail in Chapter 1 (section 1.1.10.1.).

The nature of interaction between *Acanthamoeba* and *A. butzleri* and the possible consequences of this important relation have not been studied in detail. Current knowledge is limited to the observation that *A. butzleri* can survive in *A. castellanii* for a few days (Fernandez *et al*., 2012). It is important to investigate this important interaction so that we may understand the seriousness of this relation and whether it can be a public health hazard. However, this kind of relation has been studied in closely related bacteria *Campylobacter jejuni*. It was found that *Acanthamoeba* can act as environmental reservoirs for *C. jejuni* (Axelsson-Olsson *et al*., 2005) which means that *Acanthamoeba* can provide shelter and protection to these bacteria in the environment (Vieira *et al*., 2015). Subsequently it was found that intra-amoebal *C. jejuni* could infect poultry through drinking water (Snelling *et al*., 2008).

It is an established observation that the bacterial habitable areas in the vicinity of human populations act as reservoirs for a variety of bacteria including human pathogenic bacteria (Evstigneeva *et al*., 2009). Since *Arcobacter* have been isolated from the environmental matrices (Collado and Figueras, 2011) where *Acanthamoeba* prey upon bacteria, the current study was undertaken to investigate the interaction of this predator-prey pair.

Interaction between *Acanthamoeba* and bacteria can usually be studied by investigating various parameters:

- Mechanisms involved in phagocytosis of bacteria.
- Intracellular survival of bacteria in *Acanthamoeba* (using infection assays).
- Pathogenic potential of bacteria towards *Acanthamoeba* (using plaque assay).
- Impact of intracellular survival in *Acanthamoeba* on pathogenicity of bacteria (using re-infection experiments).
- Role of two-component system activation on the virulence of bacteria towards *Acanthamoeba* (by using nicotinic acid).
- Effect of living together of bacteria and *Acanthamoeba* (using co-cultures).
- Chemotactic response of *Acanthamoeba* towards bacteria.

3.1.2. Mechanisms involved in phagocytosis of bacteria by *Acanthamoeba*

Attachment and phagocytosis are preliminary steps in the internalization of bacteria into a phagocytic cell. Phagocytosis is the engulfment of target materials which are usually in the form of bacteria or other foreign materials. The phagocytic cell forms pseudopodal protrusions to cover and grasp the prey which is then internalized into the cell body and a lysosomal assisted degradation process is initiated (Pollard *et al*., 2008). Bacteria become attached to the surface receptors of *Acanthamoeba* and are then engulfed by phagocytosis, trapping the bacteria in a phagosome which serves as a killing compartment after receiving hydrolytic enzymes from lysosomes and is then turned into phagolysosome. Phagocytosis is considered to be an actin-based process executed by polymerisation of G-actin (monomeric) into F-actin (filamentous) which allows *Acanthamoeba* to internalize bacterial food (Alsam *et al*., 2005).

Acanthamoeba has a sophisticated mechanism to phagocytose and kill its food. It was shown that *A. castellanii* can phagocytose heat-killed *E. coli* by an actin-dependent mechanism which can be blocked by phosphatidylinositol 3-kinase (PI 3-kinase) inhibitors (Alsam *et al*., 2005). Once internalized by the cell, the bacteria are grossly killed by using hydrolytic enzymes of lysosomes that can kill and digest the prey (Gildea *et al*., 2005).

The main events involved in the uptake and killing of bacteria by a phagocytic cell include uptake, internalization and phagosomal digestion. Therefore, the process of phagocytosis can be studied by blocking these steps. Attachment to the cell is usually aided by the receptors on the surface of cell that can be blocked by using different sugars like glucose, galactose and mannose (Figure 3.1-a). Since the process of phagocytosis is dependent upon the microfilaments and cytoskeletal rearrangements, therefore, any inhibitory effect on these processes can disturb the normal phagocytosis mechanism and hamper the ability of the cell to phagocytose (Alsam *et al*., 2005; Cossart and Sansonetti, 2004; Elliott and Winn, 1986). Since it is a complex phenomenon and involves various processes, a number of inhibitors are used to selectively block these processes to understand the precise mechanisms involved (Figure 3.1-b). These inhibitors are discussed below (Section 3.1.2.2.). Following ingestion of bacteria in the cell within a vacuole, acidification of phagosome is important to initiate the digestion process. Acidification is a complex process involving various triggers, therefore, use of various phagosomal acidification inhibitors is helpful in understanding the exact mechanisms involved (Figure 3.1c). The details of these is presented below (Section 3.1.2.3.).

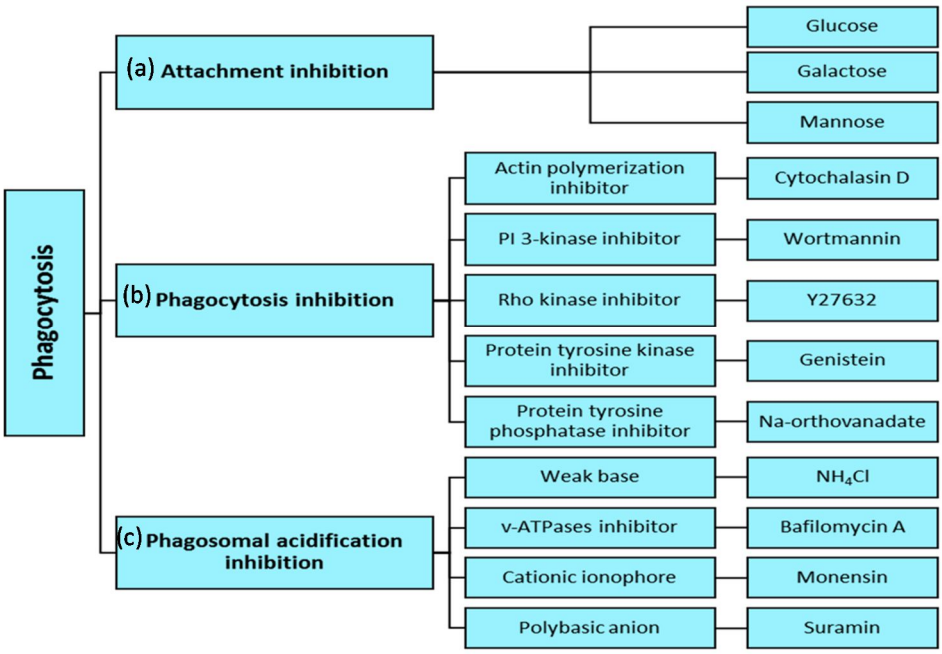


Figure 3.1: Strategies commonly used to study the various mechanisms involved in the phagocytosis of bacteria by phagocytic cells and *Acanthamoeba*. The mechanism of phagocytosis includes attachment of bacteria cell to the cell, internalization of bacteria into the cell and killing of bacteria in phagosome. Therefore, the whole process of phagocytosis can be studied in detail by using various inhibitors that selectively block these steps. (a) Attachment of bacteria to the cell is the first step and usually involves surface receptors that can be blocked by use of different sugars like glucose, galactose and mannose. (b) Internalization of bacteria into the cell is a complex process that requires involvement of various structures to aid in coordinated movement of cell to form pseudopods and trap the bacteria in a vacuole. This requires use of various inhibitors including actin polymerization (cytochalasin D), PI3-kinase (wortmannin), Rho kinase (Y27632), protein tyrosine kinase (genistein) and protein tyrosine phosphatase (Na-orthovanadate) inhibitors. (c) Similarly the process of acidification which is vital for initiating the digestion process, is complex and thus requires various acidification inhibitors to understand the correct pathways involved. These inhibitors include weak bases (Nh_4Cl), v-ATPAses inhibitor (bafilomycin A), cationic ionophore (monensin) and polybasic anion (suramin).

The various phagocytosis inhibitors that are used to investigate the phagocytosis mechanisms by phagocytic cells are discussed below:

3.1.2.1. Attachment inhibition

Mannose binding proteins (MBPs) have been shown to be important for internalization of bacteria (*E. coli*) by *Acanthamoeba* (Alsam *et al*., 2005). MBPs are also involved in attachment of *Acanthamoeba* with the mammalian cells which is the first step in their pathogenesis (Garate *et al*., 2004). Different sugars including glucose, galactose and mannose have been documented to block the sites for attachment on the cell

surface and thus play role in the attachment and thus uptake of bacteria into *Acanthamoeba* cells (Medina *et al*., 2014).

3.1.2.2. Phagocytosis inhibition

To block the internalization of bacteria into the cell various inhibitors are used to selectively understand the underlying mechanisms involved (Figure 3.16):

3.1.2.2.1. Actin polymerization inhibitors

Cytochalasin D as an actin polymerization inhibitor has long been used for this purpose (Alsam *et al*., 2005; Elliott and Winn, 1986; King *et al*., 1991). Cytochalasin D was used to study the phagocytosis process in *Legionella* by *Acanthamoeba* where it was shown to decrease the uptake of bacteria (Moffat and Tompkins, 1992). The effect of cytochalasin D on reduction of phagocytic activity and movement was also observed by (Ravdin *et al*., 1985; Stockem *et al*., 1983). Cytochalasin D was shown to reduce the uptake of *Listeria monocytogenes* by *A. polyphaga* (Akya *et al*., 2009). Similar findings were reported in case of *E. coli* (Alsam *et al*., 2005) and *A. butzleri* (Medina *et al*., 2014).

3.1.2.2.2. Phosphoinositide 3-kinase inhibitors

Wortmannin is an irreversible inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase) that inhibits actin polymerization. PI3 kinases are involved in membrane trafficking, receptor-mediated signal transduction and actin remodelling and, therefore, play important role in phagocytosis, pseudopod formation and trafficking (Cox *et al*., 1999; Downes *et al*., 2005; Wymann and Pirola, 1998). Wortmannin was found to reduce the uptake of *Listeria monocytogenes* by *A. polyphaga* (Akya *et al*., 2009).

3.1.2.2.3. Rho kinase inhibitors

Y27632 is a Rho kinase inhibitor which inhibits myosin light chain phosphorylation and cofilin phosphorylations thereby partially blocking the RhoA pathway by preventing the formation of stress fibres. Rho GTPases (RhoA, Cdc42 and Rac1) are regulators of actin polymerisations. RhoA is known to induce actin polymerisations with eventual formation of stress fibres. Y27632 was shown to partially block the RhoA pathway as manifested by reduced uptake of *E. coli* by *Acanthamoeba*

indicating the possible role of other GTPases as well (Alsam *et al*., 2005). However, this was in contrary to experiments with *A. butzleri* where the bacterial uptake was significantly reduced in a concentration-dependent manner (Medina *et al*., 2014).

3.1.2.2.4. Protein tyrosine kinase inhibitor

Genistein is a protein tyrosine kinase inhibitor that decreases the uptake of bacteria by the cell. This was observed in the case of *Acanthamoeba* for *E. coli* and *A. butzleri* (Alsam *et al*., 2005; Medina *et al*., 2014). This indicates the important role of protein tyrosine kinases in the phagocytosis by *Acanthamoeba*.

3.1.2.2.5. Protein tyrosine phosphatase inhibitor

Sodium orthovanadate is a protein tyrosine phosphatase inhibitor which increases the bacterial uptake (Alsam *et al*., 2005; Medina *et al*., 2014).

3.1.2.3. Phagosomal acidification inhibition (Figure 3.1-c)

After the phagocytosis of bacteria by the phagocytic cells like macrophages, the degradation of the prey requires phagosomal acidification (Styrt and Klempner, 1988). This process can be inhibited by using the neutralizing agents or the inhibitors of acidification. **Ammonium chloride** is a weak base which serves as a neutralizing agent by interfering with maturation of the phagosome and inhibiting phagosome–lysosome fusion. It works by penetrating the cell membrane and accumulates in acidic compartments where it neutralizes the acidic content and thereby interferes with the phagosome maturation (Hart and Young, 1991). **Bafilomycin A** is primarily a macrolide antibiotic but it is also a highly specific inhibitor for vacuolar-type proton translocating ATPases (v-ATPases) which are involved in phagosome acidification (Bowman *et al*., 1988). **Monensin** is a H^+/Na^+ ionophore which maintains a balance between the pH inside and outside of the cell and thus serves as a cationic ionophore and carrier (Prabhananda and Kombrabail, 1992). It has an inhibitory effect on the acidification and also a generalized effect on the cell organelles as well as on the intracellular transport system. **Suramin** is a polybasic anion that becomes concentrated in the lysosome. It acts by accumulating in the lysosomes and has the potential to interfere with the function of various enzymes and has been used to interfere with maturation of phagosome by preventing fusion of phagosome and

lysosome, therefore, preventing killing of bacteria (Draper *et al*., 1979; Gildea *et al*., 2005).

3.1.3. Intracellular survival of bacteria in *Acanthamoeba* (infection assays)

The assessment of intracellular survival of bacteria is usually made through infection assays. This involves infecting the cells with bacteria followed by their recovery at various time points. The first time point is usually taken within a few hours after infection while the later time points are taken for few more hours or few days. The data is represented by plotting a graph between the numbers of bacteria recovered vs. the time intervals. There are, however, various modifications in the procedure for infection assays have been adopted by different laboratories according to the nature and purpose of experiments or merely for ease of operation.

For example, Akya *et al*., (2009) studied intra-amoebaic killing of *Listeria monocytogenes* on a 24-well plate with monolayers of *A. polyphaga* that were washed thrice with saline and then inoculated with bacteria at a multiplicity of infection (MOI)=100 and at 22°C for 1h. Bacteria were then removed and the monolayers were washed again with saline thrice. Following another incubation at 22°C the cells were lysed by triton-X100 and CFUs calculated by plating lysate onto BHI agar. Abd *et al*., (2003) established co-cultures of *A. castellanii* and *Francisella tularensis* in 75 cm^2 tissue culture flasks taking 10^5 cells/mL in 50 mL of ATCC medium 712 containing 20 µg/mL tetracycline while bacteria were added at an MOI=10 followed by incubation of flasks at 30°C. For recovery of bacteria from the cells, a 3 mL sample was withdrawn and centrifuged at 300 ×g for 10 min. To reduce the extracellular burden of bacteria, the cells were washed six times with FacsWash solution followed by reconstitution in 500 µL of FacsWash solution and addition of 250 µg/mL of gentamicin for 1h at RT. The samples were then centrifuged again and an aliquot (100 µL) was spread onto the surface of the modified Thayer-Martin agar plates. Then each plate was diluted 2-fold with 0.5% sodium deoxycholate and incubated for 5 min at RT. Finally, 10-fold serial dilutions were made (10^{-1} to 10^{-4}) and plated on modified Thayer-Martin agar plates.

Rahman *et al*., (2008) studied interaction between *Aeromonas* and *Acanthamoeba* following procedure described by Abd *et al*., (2003) with the exception that instead of FacsWash solution, PBS was used and gentamicin was added at 500 µg/mL instead of 250 µg/mL. Abd *et al*., (2005) studied intracellular survival and replication of *Vibrio cholerae* O139 in *Acanthamoeba*. Co-cultures were established in 75 cm^2 culture flasks with 105/mL *A. castellanii* in ATCC medium 712 while bacteria were added at an MOI=10 and incubated at 30°C. For the recovery of intracellular bacteria, 2 mL co-culture was diluted with 8 mL PBS and centrifuged at 300 ×g for 10 min followed by three washes with PBS and reconstitution with 1 mL PBS containing 250 µg/mL of gentamicin for1 h at RT. This was then diluted with 9 mL PBS and again centrifuged. An aliquot (100 µL) of supernatant was spread onto Thiosulfate-Citrate-Bile-Sucrose (TCBS) agar plates and each pellet diluted 2-fold with sodium deoxycholate (0.1%). Then 10-fold serial dilutions (10-1 to 10-4) of sample were made and spread on TCBS agar plates and incubated at 37°C for 24h for counting CFUs.

A similar method was used by Saeed *et al* (2009) to study the interaction between *A. castellanii* and *Shigella*. Cirillo *et al*., (1994) studied intracellular growth of *L. pneumophila* in *A. castellanii*. Assay was carried out in 24-well plate having 2.5×10^5 cells per well that was left overnight at 23°C for adherence of cells with the plate. The cells were washed and incubated with saline for 1h. The infection with *L. pneumophila* was carried out at an MOI=100 followed by spinning of plate at 300 ×g to sediment the bacteria and incubated for 30 min. After washing of cells with PBS, culture media was added containing 100 µg/mL of gentamicin for 2h. The cells were then washed with PBS and then with water before incubating them with 1 mL water for 10 min. For recovery of intracellular bacteria the cells were disrupted by passing them four times through syringe needle followed by plating onto agar plates.

Since different infection assays are developed according to individual needs and every method has its own pros and cons leaving room for improvement always. One of the major improvement that can be made is switching to infection in suspension instead of plate. This is because that infection in 24-well plate is based on assumption that during infection and washing there is no loss of cells in the form of detachment and

that the infected cells are remain attached to the surface as firm enough as the uninfected ones. Practically, different isolates of *Acanthamoeba* can have different attachment potential. Infection in suspension can easily overcome this limitation. There is no loss of cells and the extracellular bacteria can easily be removed by a combination of washing and antibiotics making sure the eventual CFU count is more accurate than the one obtained in plate.

3.1.4. Pathogenic potential of bacteria towards *Acanthamoeba* (plaque assay)

A plaque assay determines the ability of bacteria to spread in a cell monolayer (Sun *et al*., 1990). The spreading potential of bacteria depends upon various factors including phagosome escape, actin-based motility and cell-to-cell passage (Portnoy *et al*., 2002). Plaque assay has been used to investigate the interaction between eukaryotic cells and bacteria (Deshayes *et al*., 2012; Portnoy *et al*., 2002; Sun *et al*., 1990). When the cells are infected by bacteria, the assay helps assess the rupture of cells by the bacteria and the level of spread of bacteria to the neighbouring cells. The cells in the form of a monolayer are infected by the bacteria at various MOIs (usually <1:1) and incubated so that bacteria become internalized into the cells. After giving plenty of time for this interaction, the cells are dyed. The dye is differentially taken up live and dead cells thus clearly differentiating the plaques that are formed as a result of the cell rupture. The greater the plaque size the greater is the degree of spread of bacteria (Tilney and Portnoy, 1989; Vazquez-Boland *et al*., 2001).

3.1.5. Impact of intracellular survival in *Acanthamoeba* on pathogenicity of bacteria (using re-infection experiments)

The fact that *Acanthamoeba* can be infected with bacteria was first reported in 1956 (Drozanski, 1956). Probably no one at that time might have realized how the apparently simple predator-prey relation between *Acanthamoeba* and bacteria can have so dynamic and significant consequences. Since that preliminary observation, the understanding of the association between *Acanthamoeba* and bacteria has expanded. It has been observed that the growth of bacteria inside *Acanthamoeba* can have dramatic changes on the bacteria in different ways. Such interaction, among other consequences, has been found to modulate pathogenic potential in some cases. For

example, this is evidenced by the presence of new outer membrane protein and fatty acid in *L. pneumophila* following internalization by *Acanthamoeba* (Barker *et al*., 1993) which were morphologically different as well (smaller in size with greater motility and resistance to chemicals) (Barker and Brown, 1995; Barker *et al*., 1992; Barker *et al*., 1995). Moreover, they had enhanced invasive capabilities towards macrophages, epithelial cells (Cirillo *et al*., 1994) and monocytes and showed more virulence for mice (Cirillo *et al*., 1999).

Similarly survival of *M. avium* in *Acanthamoeba* was reported to enhance invasiveness to cells (epithelial and macrophages), virulence to mice (Cirillo *et al*., 1997) and resistance to chlorine and antibiotics (Miltner and Bermudez, 2000; Whan *et al*., 2006). Likewise, intracellular survival of some bacteria has been shown to enhance the tolerance of these bacteria to chlorination which include coliforms (*Escherichia coli, Enterobacter cloacae, E. agglomerans, Citrobacter freundii, Klebsiella pneumoniae, K. oxytoca*) and other bacterial pathogens (*Legionella gormanii, S. typhimurium, C. jejuni, Yersinia enterocolitica, Shigella sonnei*) (King *et al*., 1988). A number of *Acanthamoeba*-surviving bacteria have also been demonstrated to survive in cysts including *L. pneumophila* (Winiecka-Krusnell and Linder, 1999), *Pseudomonas aeruginosa* (Marciano-Cabral and Cabral, 2003), *Vibrio cholerae* (Li *et al*., 2006), *M. avium* (Adekambi *et al*., 2006), *M. bovis* (Taylor *et al*., 2003), *Acinetobacter baumanii* (Cateau *et al*., 2011). However, this has not been investigated in context with important emerging human pathogen *A. butzleri*, which is one of the aims of this study.

3.1.6. Role of two-component system activation on the virulence of bacteria towards *Acanthamoeba* (by using nicotinic acid)

A usual way of responding to the external stimuli, by bacteria, is via a two-component signal transduction system. Although in the majority of cases it is not clear how precisely the cues trigger autophosphorylation and then phosphorelay, however, different environmental stimuli are known to actually activate these systems (Calva and Oropeza, 2006). It has been observed that the pyridine derivative nicotinic acid (NA) can modulate the activity of microbial two-component systems and, consequently, regulate the genes and phenotypes that are governed by these regulatory

proteins (Edwards *et al*., 2013). Although the exact mechanisms are not clear, however, NA can modify the transcriptional and phenotypic characteristics of different types of microbes. For example, in *Bordetella pertussis* exogenous NA alters the expression of a wide variety of genes and in turn the virulence (Cotter and DiRita, 2000; Cummings *et al*., 2006; McPheat *et al*., 1983; Schneider and Parker, 1982). Similarly in *E. coli*, NA induces a number of changes including motility, outer membrane porin protein, and the alcohol dehydrogenase protein (Han *et al*., 1999; Leonardo *et al*., 1996; Utsumi *et al*., 1994). In *L. pneumophila*, at 5 mM, NA tends to trigger various premature phenotypic traits including evasion of lysosomal degradation, motility, cytotoxicity toward macrophages and sodium sensitivity, while a total of 213 genes were found to be altered by the NA pre-treatment (Edwards *et al*., 2013).

The study of the role of NA in the virulence of *A. butzleri* can help understand the effect of environmental change on the behaviour of these bacteria and the outcome of their interaction with *Acanthamoeba* which may explain how changes like this can also play a role in adaptation of bacteria to intracellular environment.

3.1.7. Effect of living together of bacteria and *Acanthamoeba* (co-cultures)

The co-culture experiments have been used to study the impact of culturing *Acanthamoeba* and bacteria together to assess the effect on mutual growth. For the experimental design, the co-cultures have been used by simply culturing both the organisms together or by separating them by a filter paper in a parachamber with very small pore size. In the latter case, amoebae and bacteria don't have chance to come in contact with each other while they share the medium they are in so they can only chemically communicate with each other unlike the direct method where both are present physically in the same medium. Both the direct and parachamber co-culture methods have been used to study the effect of co-culture of *Acanthamoeba* and different bacteria including *M. avium, E. coli*, *L. pneumophila*, *T. equigenitalis* and *T. asinigenitalis.* These are usually carried out in 24-well plates at different MOIs (50:1, 100:1) followed by daily observation for a few days (Allombert *et al*., 2014; Steinert *et al*., 1998).

3.1.8. Chemotactic attraction of *Acanthamoeba* towards bacteria

Chemotaxis was first described by a German botanist TW Engelmann (1881) followed by others including WTP Pfeffer (1884) in bacteria and HS Jennings (1906) in ciliates (Ben-Menahem, 2009). Chemotaxis is an oriented response to a stimulus (Wilkinson, 1982) and can be defined as the movement of cells in response to the chemicals which may be positive (in which case the cells are attracted towards the source) or negative (whereby the cells are repelled away from the chemotactic substances). It is an ancient form of cell communication that involves sensing of a chemoattractant gradient and plays a crucial role in different functions such as body development, infections, inflammation defence and feeding. Phagocytes, including neutrophils and macrophages, detect invading bacteria through chemotactic stimuli via chemicals excreted by the bacteria (Devreotes and Zigmond, 1988). To arrive at a site of infection, neutrophils detect a series of chemo-attractant signals both produced by the bacteria and by other host cells. The phagocytes integrate these signals through intracellular signalling pathways favouring some chemo-attractants over the others (Heit *et al*., 2002). As with mammalian phagocytic cells that use this approach for locating intruding bacteria in the body, chemotaxis serves as an important aid in locating bacteria in soil (Hugli, 1989). Preliminary studies of chemotaxis by *Acanthamoeba* indicated the involvement of a signal (chemotactic factor), either in bacteria or released by the bacteria, in attracting amoeba (Tharavanij, 1965). *Naegleria fowleri* was shown to exhibit chemotaxis towards bacteria as well as chemotactic peptide (Marciano-Cabral and Cline, 1987). Chemotaxis was also demonstrated in *Entamoeba histolytica* towards *E. coli* (Urban *et al*., 1983).

An extensive study was carried out by Schuster *et al*., (1993) to evaluate the chemotactic response to *Acanthamoeba* towards various Gram+ve (*Bacillus cereus, Clostridium tetani, Micrococcus luteus, Staphylococcus aureus*) and Gram-ve (*Chromobacter violaceum, Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas fluorescens, S. typhimurium, Serratia marcescens, Shigella boydii, Yersinia enterocolitica*) bacteria and their products using migration of amebae under the agar. It was interesting to find that amoebae showed positive chemotaxis not only to normal prey bacteria but also to the bacteria they do

not normally ingest because of their pigment contents. The bacterial lipopolysaccharide and lipoteichoic acid components of cell wall failed to induce a chemotactic response. Furthermore, formyl-methionyl-leucyl-phenylalanine (chemotactic peptide) demonstrated a positive chemotactic response but it was blocked by the antagonist peptides. It was concluded that the chemotactic response by amoebae is dependent upon the receptors on their surface.

According to the direction of cells movement, chemotaxis can be classified as positive or negative. Chemical substances which attract cells toward it are called chemo-attractants while the others which cause the cells to move away from them are called chemo-repellents. Positive chemotaxis results in accumulation of cells in the region of higher chemo-attractant concentration while negative chemotaxis results in scattering of cells away from the chemo-repellent. Motility of cells is often studied by looking at cells moving on a cover slip in a liquid environment where they face little resistance and that is not the case for them in real life. The test is usually performed using the under-agar technique where amoebae have to move under the agar in a petri plate (Figure 3.2). Since the amoebae moving in soil or neutrophils penetrating through the endothelium of capillary have to move under resistance, therefore, under-agar assay simulates natural conditions and is rapid and simple method (Nelson *et al*., 1975).

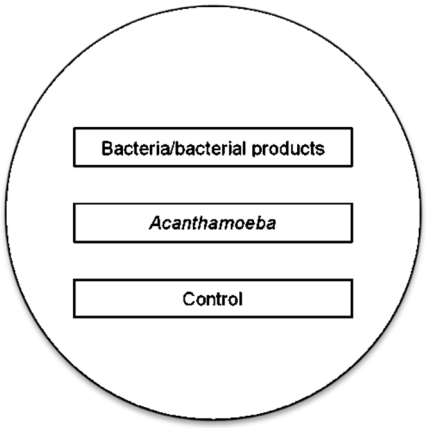


Figure 3.2: Layout of the agar plate for under-agar experiment to study the chemotactic response of amoebae towards bacteria. Three troughs are cut out in parallel in an agar plate. The centre trough is used for loading the amoebal suspension while one of the adjacent troughs is used as test and contains bacteria or the bacteria products to study and the other trough is used for the control. Amoebae move in response to the chemotactic attraction or repulsion towards or away from the test trough. The movement of amoebae is recorded against time and the information if further processed for quantitative analysis.

The chemotaxis experiments were conducted using under the agar method to understand the chemotactic response of *Acanthamoeba* towards *A. butzleri* in order to evaluate the ability of these bacteria to be located by amoebae as food source in the environment.

3.1.9. Objectives of the study

This chapter was aimed at exploring the interaction between *Arcobacter* and *Acanthamoeba* at the molecular and cellular level in a robust fashion addressing a number of aspects of this important relation. The main objectives were to study:

- the mechanisms involved in phagocytosis of *A. butzleri*. This included the mechanisms of attachment and entry (using various phagocytosis inhibitors) of *A. butzleri* into *Acanthamoeba* cells.
- the intracellular survival and time course of infection of *A. butzleri* in *Acanthamoeba* (using infection assays).
- the pathogenic potential of *A. butzleri* towards *Acanthamoeba* in terms of spread of bacteria from infected cells to surrounding cells in monolayer (using plaque assay).
- the impact of intracellular survival in *Acanthamoeba* on virulence of *A. butzleri* (using re-infection experiments).
- the role of two-component system activation on the virulence of *A. butzleri* towards *Acanthamoeba* (by using nicotinic acid).
- the mutual effect of co-culture of *Acanthamoeba* and *A. butzleri* on each other.
- the effect of *A. butzleri* conditioned medium on *Acanthamoeba*.
- the capability of *Acanthamoeba* to locate *A. butzleri* as measured by chemotactic attraction of *Acanthamoeba* towards *A. butzleri*.

3.2. Materials and Methods

3.2.1. Culture media

AX2+ media was used for axenically culturing *Acanthamoeba* strains while VD media was used for growing *A. butzleri* strains as described in Chapter 2 (Section 2.2.1.).

3.2.2. Bacterial strains, culture and viable counts

A. butzleri strains used included ED-1 (WT), ArcoL (WT), RM 4018 (WT) and GFP expressing ED-1_GFP. The ED-1 strain is a microaerobic exoelectrogenic eplisonptoteobacterium that was originally isolated from the electrode of a microbial fuel cell while Arco-L was also isolated from the liquid phase of the same cell (Fedorovich *et al*., 2009). The RM-4018 strain is identical to the ED-1 strain in 16S rRNA sequence (Miller *et al*., 2007). These strains were kindly provided by Dr Bruce Ward, School of Biological Sciences, University of Edinburgh. Experiments with the WT strains were carried out in CL-2 laboratory (Hugh Robson Building, University of Edinburgh) while the work with GFP bacteria was performed in CL-2 laboratory designated for GMOs (Darwin Building, University of Edinburgh). Bacteria were cultured in Vandamme media at 30ºC under microaerophilic conditions (Knighton *et al*., 2013). Viable counts of bacteria were performed as described in Chapter 2 (Section 2.2.2.).

3.2.3. Growth curves and calibration curves of *A. butzleri*

The growth curves for the bacteria were determined using a method described by Letek *et al*., (2010). Overnight cultures at stationary phase were used to prepare the start culture that was diluted extensively to achieve an OD_{405} of 0.05. Bacteria were grown in VD media without gentamicin as well as in various concentrations of gentamicin (200, 100, 500 and 10 µg/mL) at 30ºC in a plate reader (PowerWave XS, BioTek, USA) with constant shaking in flat-bottom 96-well plate. The wells were filled with 200 µL volume of the cultures in triplicate. Readings were automatically made at 30 min intervals. The graphs were manually constructed from the readings so obtained (ODs vs time).

The calibration curves of *A. butzleri* strains ED-1, Arco-L and RM-4018 were determined by taking bacteria at various ODs (1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1). Ten-fold serial dilutions were made and plated onto VD agar plates for colony count. Three replicated were carried out for all the strains. Graphs were plated for each of the three strains for CFUs/mL vs OD_{405}.

3.2.4. Suitability of detergents for lysis of *Acanthamoeba* trophozoites

The suitability of two detergents triton and sodium deoxycholate was assessed to lyse the *Acanthamoeba* cells and recover the bacteria. Sodium deoxycholate was used at concentrations 0.5%, 0.25% and 0.05% while triton was used at 0.1%, 0.05% and 0.025%. The washed *Acanthamoeba* Neff cells were suspended separately in various concentrations of sodium deoxycholate and triton for 5 min. the cells were monitored directly under the microscope for cell lysis. Cells were also vortexed briefly to aid in lysis after the treatment. The ability of these detergents to recover the maximum number of bacteria was also measured by infecting the *Acanthamoeba* Neff cells with *A. butzleri* ED-1 at an MOI of 100:1 followed by gentamicin treatment for 1h as for an infection assay. The cells were then lysed with various concentrations of sodium deoxycholate and triton for comparison of recovery of bacteria following lysis. The effect of triton on the viability of *A. butzleri* was also measured by exposing *A. butzleri* cells to triton at 1%, 0.5% and 0.1% for 5 min along with untreated bacteria cells. The bacterial count was then performed by making 10-fold serial dilutions and plating on the VD agar plates.

3.2.5. Evaluation of the efficacy of gentamicin to kill *A. butzleri* in 1h

The effect of gentamicin on killing a large number of *Arcobacter* in 1h was studied by exposing the bacterial culture (~5×10^5/mL) with 200 µg/mL of gentamicin for 1h followed by 10-fold serial dilutions and plating on the VD agar plates for colony counts in parallel with the non-treated bacterial culture as control.

3.2.6. Role of sugars in phagocytosis of *Arcobacter* by *Acanthamoeba*

The role of sugars in phagocytosis of *Arcobacter* by *Acanthamoeba* was studied by treating the washed *Acanthamoeba* cells separately with glucose, galactose and mannose at different concentration levels (10, 50 and 100 mM) for 1h. Three

different strains of *A. butzleri* were used (ED-1, Arco-L and RM-4018) along with Neff and three other *Acanthamoeba* soil isolates (Eco-B01 (T4-A), Ent-M02 (T4-B) and Arc-E07 (T4-E)). The infection of the cells with bacteria was proceeded as for infection assay (Akya *et al*., 2009; Alsam *et al*., 2005; Medina *et al*., 2014).

3.2.7. Role of phagocytosis inhibitors on uptake of *Arcobacter* by *Acanthamoeba*

The effects of various inhibitors on the phagocytosis of *Arcobacter* by *Acanthamoeba* were studied. As for sugars, the washed *Acanthamoeba* cells were treated with varying concentrations of inhibitors for 1h followed by infection with *A. butzleri* strains (ED-1, Arco-L and RM-4018). The inhibitors used included cytochalasin D, wortmannin, sodium orthovanadate, ammonium chloride, monensin, bafilomycin A and suramin (Akya *et al*., 2009; Alsam *et al*., 2005; Medina *et al*., 2014).

3.2.8. Infection assays for *Acanthamoeba* and *Arcobacter*

The gentamicin infection assay as described by Van Langendonck *et al*., (1998) was used to study the intracellular survival of *A. butzleri* in *Acanthamoeba* with modification. The infection was performed in suspension rather than 24-well plate to avoid any loss of cells due to detachment from the surface of plate.

3.2.8.1. Preparation of *Acanthamoeba* suspension

Acanthamoeba cells were grown axenically to near confluence in AX2+ media in 75 cm^2 cell culture flasks. A day before infection the flask media was replaced with fresh AX2+. On the day of infection, the media in the flask was removed and the cells monolayer washed twice with NS. Approximately 10 mL more NS was added and the cells were scrapped off the surface gently with the help of a sterile cell scrapper. The cells were then transferred to a sterile tube. The tube was gently rolled out in palms to distribute the cells thoroughly. A 50 µL cell suspension was sucked up and mixed with equal volume of trypan blue an Eppendorf tube. The cells were counted under the inverted microscope (Leica, DM IRB, Germany) with the help of cell counting chamber. The number of cells in the tube was adjusted to 4×10^5/mL.

3.2.8.2. Preparation of bacterial suspension

A. butzleri cultured to OD_{405}=1.0 were washed twice with PBS and after reconstitution the OD was measured. Calculations were made to adjust MOI=100 and the resulting suspension was ready for infection.

3.2.8.3. Infection of *Acanthamoeba* cells with bacteria

The calculated volume of bacterial suspension was added to the *Acanthamoeba* cell suspension (MOI=100:1) in 15 mL tube and the tube was gently inverted to mix the contents. Infection of the *Acanthamoeba* cells was allowed to proceed for 1h followed by centrifugation at a low speed of 800 rpm for 5 min to sediment the *Acanthamoeba* cells leaving the bacteria floating on the top. The supernatant was discarded and the cells were washed three times with NS to remove most of the bacteria. The cells were then re-suspended in AX2+ containing 200 µg/mL of gentamicin and allowed to interact for 1h to kill the remaining extracellular bacteria. After this time a 1 mL aliquot of cells was taken out. The cells were centrifuged and washed twice. The cells were then lysed by the addition of 100 µL of triton X-100 (0.1%) for 5 min followed by dilution with 400 µL of VD and brief vortexing. From this lysate, 10-fold serial dilutions were made and plated onto the VD agar plates. The plates were incubated at 30ºC overnight under microaerophilic conditions so that the *Arcobacter* can grow and form visible colonies.

3.2.9. Plaque assay

The plaque assay was performed as described by (Deshayes *et al*., 2012) with some modifications. *A. castellanii* Neff and *A. butzleri* ED-1 were used for the plaque assay. Neff cell suspension (1×10^6cells/mL) was prepared as described for the infection assay above. The cell suspension was added to the wells of 6-well plate by pouring 2 mL in each well. The plate was left at 23ºC overnight for the cells to adhere firmly. *A. butzleri* ED-1 (OD_{405}=1.0) were washed twice with PBS and finally reconstituted in VD. Their OD_{405} was measured to calculate the cell number per mL. For infection of cells, the media from each well was removed and the cell monolayers were washed once with NS. Fresh VD was added to all the wells followed by the bacterial suspension to attain the final MOIs of 100:1, 10:1, 1:1 and

0.1:1 in the designated wells while the control well only received VD. The plate was spun at 1500 rpm for 3 min and then incubated at 23ºC for 1h. The overlying bacterial suspension was sucked out and the wells were washed with three times with NS. Fresh AX2+ with 200 µg/mL gentamicin was added to each well (2 mL) and the plate was again left to incubate for 1h. After that time the media from the well was removed. Molten 1% agar in AX2+ that had been cooled down (fair-to-touch) containing 10 µg/mL gentamicin was poured onto the monolayers (2 mL) and allowed to solidify. The plate was secured with paraffin to reduce evaporation and covered in aluminium foil to incubate up to 72h. The cells were stained with phenol red and the monolayers viewed under an inverted microscope for any plaques formed.

3.2.10. Re-infection (of *Acanthamoeba* with *A. butzleri*) experiments

These were performed using a method described by Cirillo *et al*., (1999) with some modifications. As illustrated in Figure 3.3, the re-infection experiments were conducted with an objective to repeatedly infect the *Acanthamoeba* (Neff) with *Arcobacter* (ED-1) bacteria (using the infection procedure-section 3.2.8.) followed by recovery of bacteria from *Acanthamoeba* cells, and using these recovered bacteria to undertake another infection. A total of 15 re-infections were undertaken. For each re-infection experiment bacteria were recovered from *Acanthamoeba* by cell lysis using triton and then spread onto the VD agar plates that were kept at 30ºC under microaerophilic conditions to allow the bacteria to grow and form colonies. These bacterial colonies were then cultured in VD media (section 3.2.8.2.) and used for another re-infection experiment.

The bacteria recovered at time point 48h (or later) of infection assay were used for the next re-infection. Pathogenicity of the recovered bacteria was assessed after each re-infection by undertaking a complete infection using recovered bacteria and Neff, taking readings at day-0, -1, -2, -3, -4, -5, -6, -7, -14, -21, -28, -35, -42 and -49. The effect was assessed by observing the increase in number of bacteria recovered at each time point and the longevity of the recovery of bacteria from the Neff cells compared with the WT ED-1 bacteria.

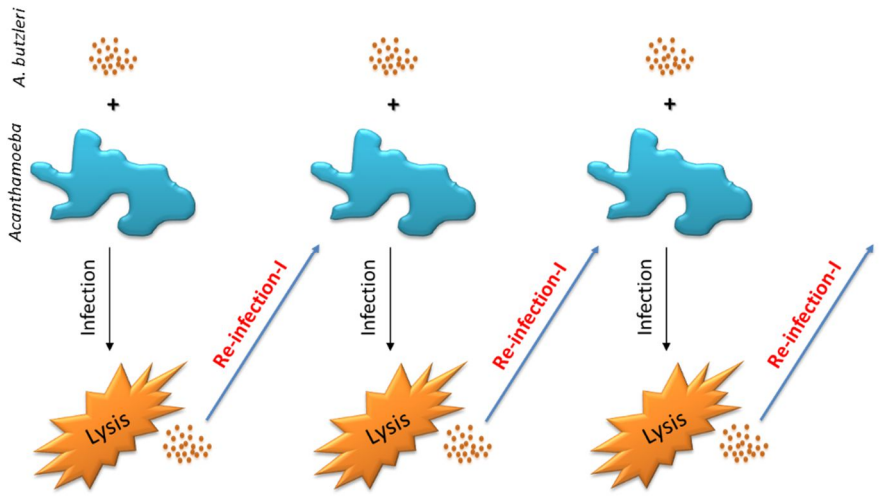


Figure 3.3: The strategy used for the re-infection experiments. Each infection was performed at an MOI=100:1 and the recovered bacteria were used for the next infection. The process of re-infection was repeated for 15 cycles. The purpose of these experiments was to assess whether intracellular survival of *A. butzleri* in *Acanthamoeba* is associated with modulating virulence properties of these bacteria.

3.2.11. Effect of nicotinic acid treatment on *A. butzleri* virulence

Bacteria were grown in normal VD media with added nicotinic acid at a final concentration of 5 mM concentration (negligible change in the pH). *Arcobacter* were cultured as usual to OD_{405}=1.0 and washed three times with PBS before using them for infection. Neff cells were washed three times with NS before scrapping them out of the flask. The infection was carried out in suspension at an MOI of 100:1. After 1h of infection the cells were centrifuged at very low speed (800 rpm) to sediment the *Acanthamoeba* cells but not the bacterial cells. The supernatant was discarded and the cells were washed three times by centrifugation to eliminate most of the extracellular bacteria. AX2+ media with gentamicin (200 µg/mL) was added for 1h to reconstitute the washed *Acanthamoeba* cells and to kill the remaining extracellular bacteria. The solution was replaced by a fresh media containing low levels of gentamicin (10 µg/mL) as a maintenance dose. A 1 mL aliquot was aspirated from both the infections (control and nicotinic acid treated). The cells were washed and lysed with triton 0.1% to recover the internalized bacteria that were then plated onto the VD agar plates for colony counts. After 1h of infecting the *Acanthamoeba* cells with the treated or non-treated bacteria, the cells were closely monitored under the microscope for counting the number of infected and non-infected cells as well as

those lysed. Random microscope fields were selected and a total of 100 cells were observed in each case for the evidence of motile *Arcobacter* in phagosomes.

3.2.12. Co-cultures of *Acanthamoeba* and *Arcobacter*

3.2.12.1. Infection on agar overlaid with live *Arcobacter* ED-1

In this setup, *Acanthamoeba* and *Arcobacter* were co-cultured on a non-nutrient agar plate with overlaid live washed *A. butzleri* ED-1 in saline at an OD of 1.0 (Figure 3.4). Low levels of VD media ($1/10^{th}$ diluted) were used to sustain the bacteria. *Acanthamoeba* were spotted (20 µL) with final concentrations of 2×10^2, 2×10^3, 2×10^4 and 1×10^5. The plate was immediately monitored under the microscope and time lapse photography used to record those events taking place.

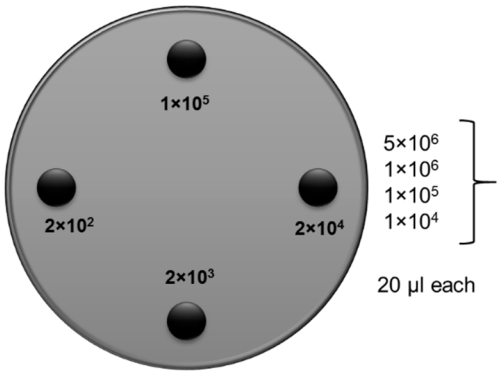


Figure 3.4: Setup of the co-culture of *Acanthamoeba* (Neff) and *Arcobacter* (ED-1) on agar plate overlaid with live *Arcobacter* with low levels of nutrients (VD) to sustain viability. Neff at various concentration levels were spotted onto the agar plat at different points to observe qualitatively the effect of co-culture.

3.2.12.2. Impact of co-culture on *Acanthamoeba* and *Arcobacter* in liquid media

The purpose of these experiments was to assess whether any particular set of growth and environmental conditions can affect the co-existence of these organisms. The effect of co-culture of *Acanthamoeba* and *Arcobacter* was studied qualitatively and quantitatively under a number of varied growth and culture conditions using different aeration (aerophilic vs. microaeophilic), different temperatures (30°C vs 22°C) and different growth media (NS, VD and AX2+) as shown in Figure 3.5.

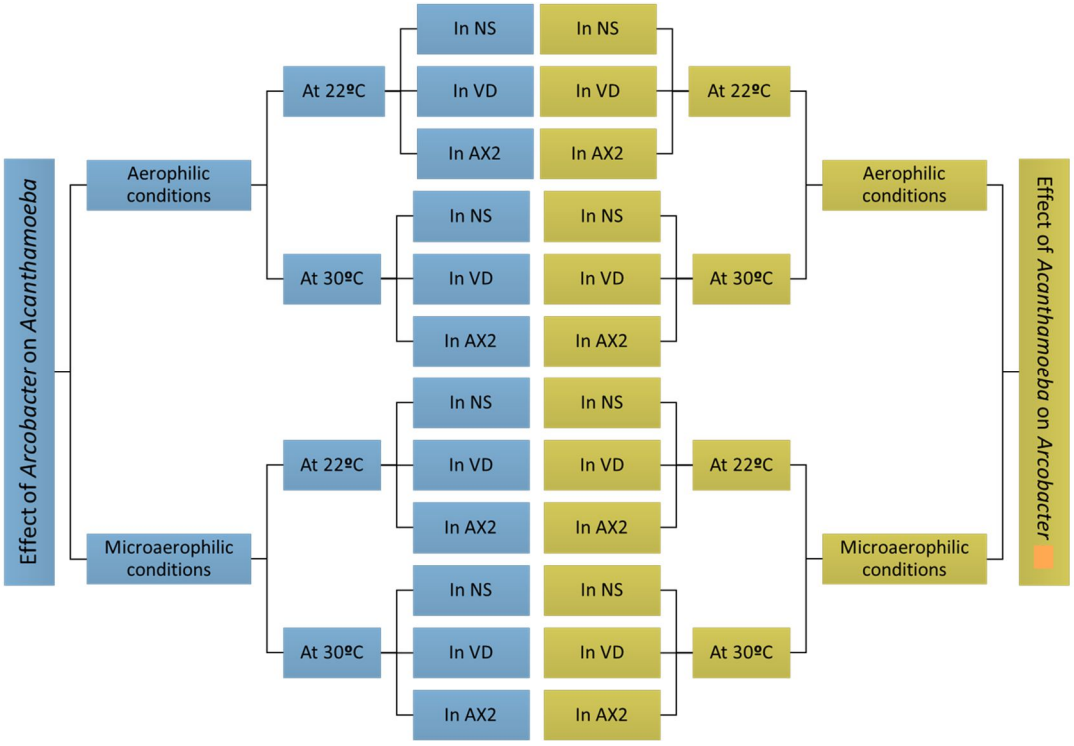


Figure 3.5: The experimental layout of the co-culture experiments with *A. castellanii* Neff and *A. butzleri* ED-1. The interaction between the two organisms (effect of *Arcobacter* on *Acanthamoeba* and effect of *Acanthamoeba* on *Arcobacter*) was studied under a variety of culture conditions with different aeration (aerophilic vs microaeophilic), temperature (30°C vs 22°C) and growth media (NS, VD and AX2+).

3.2.13. Effect of *Arcobacter* on excystment and encystment of *Acanthamoeba*

The effect of excystment followed by encystment of *Acanthamoeba* by *Arcobacter*-grown media was studied by culturing (in suspension) *A. castellanii* Neff in growth media with and without added media in which *A. butzleri* ED-1 was grown (*A. butzleri* conditioned media or *Ab*CM). Different controls were also used including *Ac* Neff in AX2+; *Ac* Neff in (AX2+)+*Ab*CM(VD); *Ac* Neff in *Ab*CM (VD); *Ac* Neff in (AX2+)+VD; *Ac* Neff in VD.

The *Ab*CM was prepared by culturing *Ab* in VD media overnight under microaerophilic conditions at 30ºC with constant shaking. The bacteria were pelleted down by centrifugation and the supernatant was filtered through 0.22 µm syringe filter.

The cysts were split into five different 50 mL tubes. These were centrifuged and then washed with NS twice. The cysts were finally reconstituted in the respective media. The cysts were counted in a counting chamber and the final number was adjusted to 5×10^5/mL per tube (50 mL) with a total of 10 mL volume. The tubes were incubated at 22ºC. The cysts/trophozoites were counted daily.

3.2.14. Chemotactic response of *Acanthamoeba* towards *A. butzleri*

To study the chemotactic response of *Arcobacter* towards *Acanthamoeba*, axenically grown *A. castellanii* Neff and Arc-NB03 which was a soil isolate (T2) were used while two strains of *A. butzleri* i.e. ED-1 and Arco-L were used that were grown in VD media to OD_{405}=1.0. For the preparation of plates, 2% agar plates were prepared in NS (30 mL in each plate) and dried overnight. Three sample troughs (40×4 mm) were cut out 20 mm apart with the help of a surgical blade and using the template (Figure 3.6). Care was taken not to scratch the bottom of the well to avoid any effect on cell movement during the experiment.

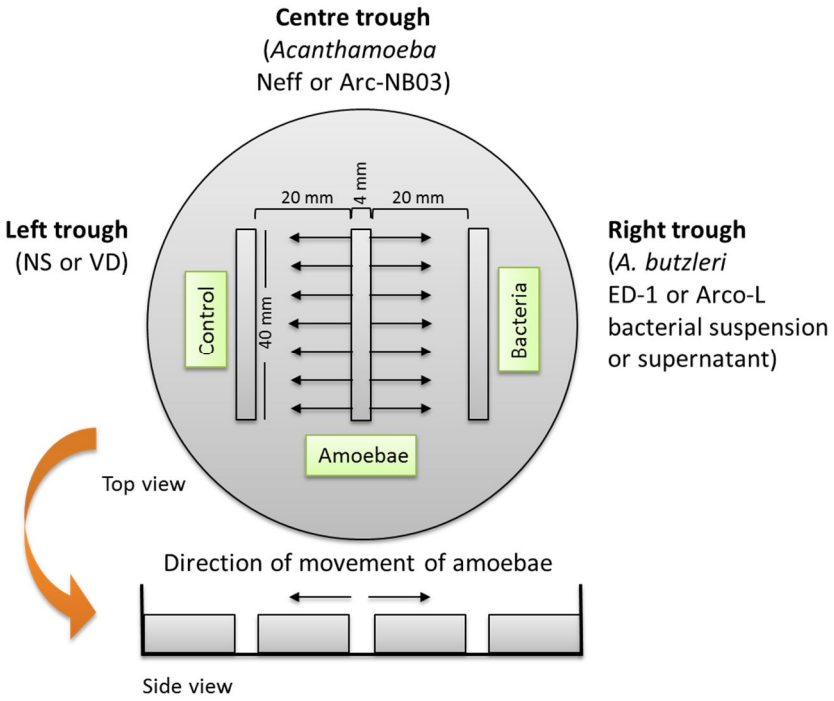


Figure 3.6: The layout of the agar plate prepared for the chemotaxis experiment (under-agar method) showing the top and side view of the plate. Three troughs were cut out (40×4 mm) and 20 mm apart. The central trough was for amoebae while the other ones are for test (bacteria, bacterial products) and control. Small arrows represent the direction of movement of amoebae.

The experimental layout for the chemotaxis experiment between *Acanthamoeba* and *A. butzleri* is shown in Table 3.1. For *Acanthamoeba* two strains (Neff and Arc-NB03) were used while for *A. butzleri* the strains used were ED-1 and Arco-L. The *Acanthamoeba* was always kept in the middle trough. For control either NS or VD media was used while for test trough bacteria in suspension or the bacteria-grown medium (supernatant) was used either non-heated or heated.

Table 3.1: Experimental layout for the chemotaxis experiment setup to study the chemotactic response of *A. butzleri* towards *Acanthamoeba*. Two different strains of both *Acanthamoeba* (Neff and Arc-NB03) and *A. butzleri* (ED-1 and Arco-L) were used under three different test conditions for bacteria i.e. bacteria in suspension (in NS), bacterial supernatant (bacteria-grown media) non-heated and bacterial supernatant heated. The controls used for each combination of experimental condition are also shown.

***Acanthamoeba castellanii* Neff**			***Acanthamoeba* Arc-NB03 isolate**		
Control (left trough)	***Acanthamoeba* (centre trough)**	**Test (right trough)**	**Control (left trough)**	***Acanthamoeba* (centre trough)**	**Test (right trough)**
NS	Neff	*A. butzleri* ED-1 suspension in NS	NS	Arc-NB03	*A. butzleri* ED-1 suspension in NS
NS	Neff	*A. butzleri* Arco-L suspension in NS	NS	Arc-NB03	*A. butzleri* Arco-L Suspension in NS
VD	Neff	*A. butzleri* ED-1 supernatant (non-heated) in VD	VD	Arc-NB03	*A. butzleri* ED-1 supernatant (non-heated) in VD
VD	Neff	*A. butzleri* Arco-L supernatant (non-heated) in VD	VD	Arc-NB03	*A. butzleri* Arco-L supernatant (non-heated) in VD
VD	Neff	*A. butzleri* ED-1 supernatant (heated) in VD	VD	Arc-NB03	*A. butzleri* ED-1 supernatant (heated) in VD
VD	Neff	*A. butzleri* Arco-L supernatant (heated) in VD	VD	Arc-NB03	*A. butzleri* Arco-L supernatant (heated) in VD

The agar plugs were carefully removed so as not to disturb the remaining agar which may create a gap underneath that can interfere with the results. The central well was used for amoebae while one of the lateral wells was used for the test chemo-attractant (bacteria, bacterial products or a chemical) and the other one was used as control (NS, VD media, or solvent used for test substance). Each trough could hold liquid upto 0.8-1.0 mL. For the experiment, each plate was loaded with control and

test solutions while saline alone was added to the central well and the plate left at RT for an hour to allow a gradient to establish. After that, saline was removed from the central well and washed amoebae in NS were added. The plate was sealed with paraffin film, covered with aluminium foil and kept in a dark place at RT.

Amoeba migration under agar was monitored and recorded hourly using an inverted light microscope (Leica DM IRB, Germany) connected with a computer operated camera (Canon EOS 1100D, UK). The distance travelled by amoebae from the edge of the well outwards (in either direction i.e. towards test and control well) was measured and graphs were plotted between the distance travelled by amoebae against the time taken along with linear regression line for this movement using Microsoft Excel. This provided an overview of the trend of amoeba movement towards different stimuli. For the statistical comparison of regression lines, GraphPad Prism6 software was used employing analysis of covariance (ANCOVA). It compares slopes first where it calculates *p*-value (two-tailed) at 95% confidence level, testing the null hypothesis that the slopes are all identical. Therefore, a *p*-value lesser than 0.05 indicates rejection of null hypothesis and hence the lines will be significantly different in this case.

3.3. Results

3.3.1. Growth pattern of *A. butzleri* strains

The growth pattern of various strains of *A. butzleri* in VD media and the effect of various concentration of gentamicin on the viability of these strains was assessed (Figure 3.7). All the strains used (ED-1, Arco-L, RM-4018 and ED-1_GFP) had similar growth pattern characterized by a lag phase of nearly 2h followed by a sharp log phase and the stationary phase of 10h. The purity of cultures was confirmed at the end of each experiment. The information obtained from the growth curves was important as it indicated that the mid-log phase, where bacteria are at maximum health, existed at OD_{405}~0.8-1.0 and, therefore, bacteria at this OD were used for experiments. All the strains were sensitive to gentamicin as no growth was observed in any of the strains even at the lowest concentration (10 µg/mL).

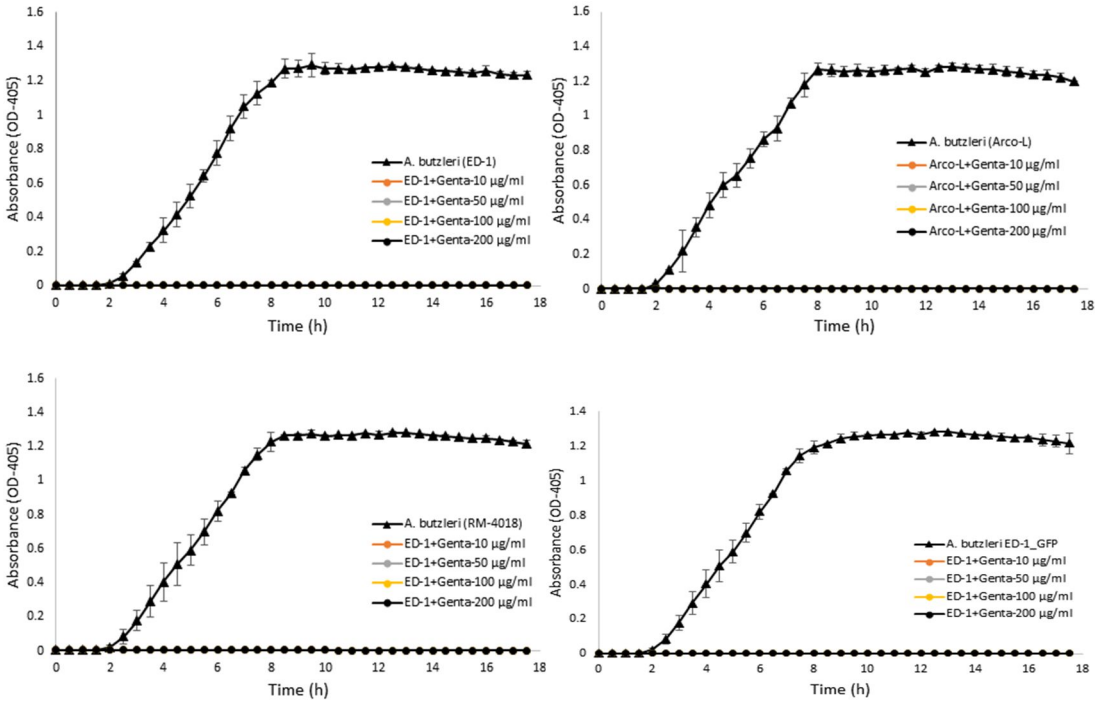


Figure 3.7: Growth curves of *A. butzleri* ED-1, Arco-L, RM-4018 and ED-1_GFP (ED-1+pJK1 expressing GFP) in Vandamme media (VD) without and with gentamicin at various concentrations (10, 50, 100 and 200 µg/ml) at 30ºC with continuous shaking in 96-well flat bottom plate at 200 µL/well in the plate reader. The initial inoculum was taken from an overnight culture of *A. butzleri* in stationary phase. The results represent mean±SD of two independent experiments.

3.3.2. Setting up of experimental conditions

3.3.2.1. Calibration curves of *A. butzleri* strains

The calibration curves of *A. butzleri* strains indicated a good linear relation between the OD and the CFUs of bacteria (Figure 3.8) over a wide range of OD (0-1.0). Linear regression lines were drawn for each graph and used for enumeration of bacteria at any specific OD for subsequent experiments.

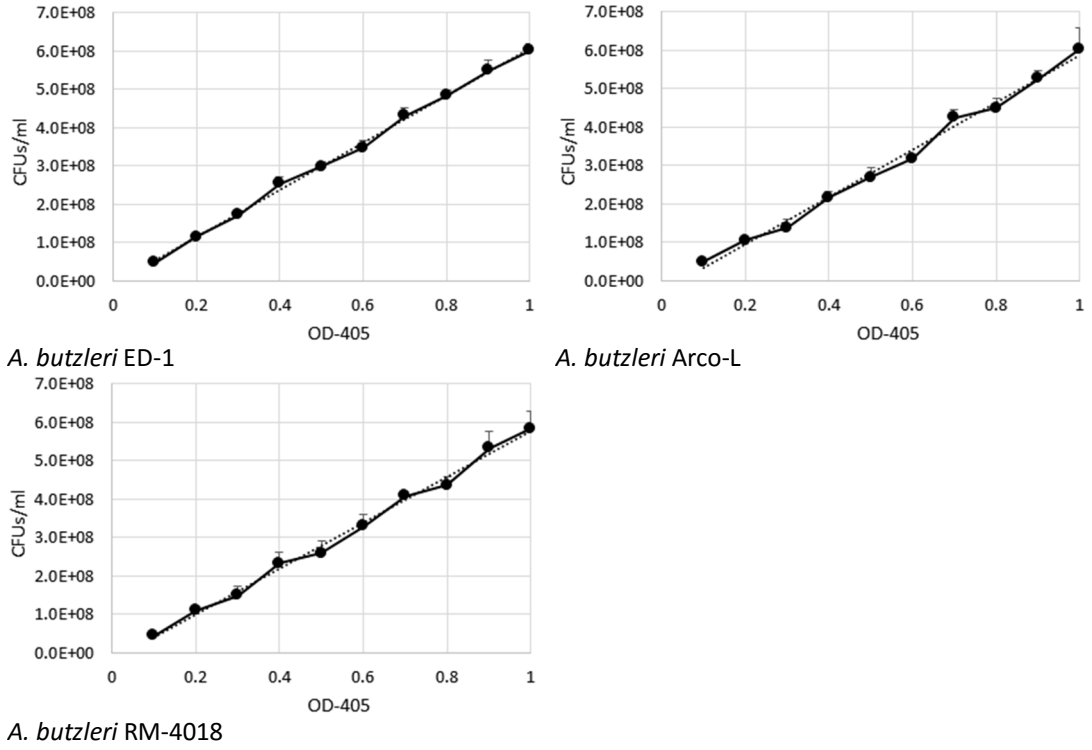


Figure 3.8: Calibration curves for *A. butzleri* ED-1, Arco-L and RM-4018 strains. The bacteria were grown in VD media at 30ºC under microaerophilic conditions with constant shaking. The bacteria at various ODs were diluted and plated onto VD agar plates for colony counts. The data represents mean±SE of three independent experiments. The dotted lines are best curve fits for each calibration curve.

3.3.2.2. Suitability of detergents for lysis of *Acanthamoeba* trophozoites

Suitability of sodium deoxycholate and triton X-100 was determined to find appropriate detergent and the concentration for the lysis of *Acanthamoeba* cells to recover internalized bacteria. Sodium deoxycholate at all the concentration levels was unable to recover maximum number of bacteria (Figure 3.9-a) in comparison to triton at 0.1% (Figure 3.9-b). Also the effect of triton on the viability of *A. butzleri* was

tested. The 0.1% concentration of triton was found to be suitable for recovery of bacteria without affecting their viability (Figure 3.9-c).

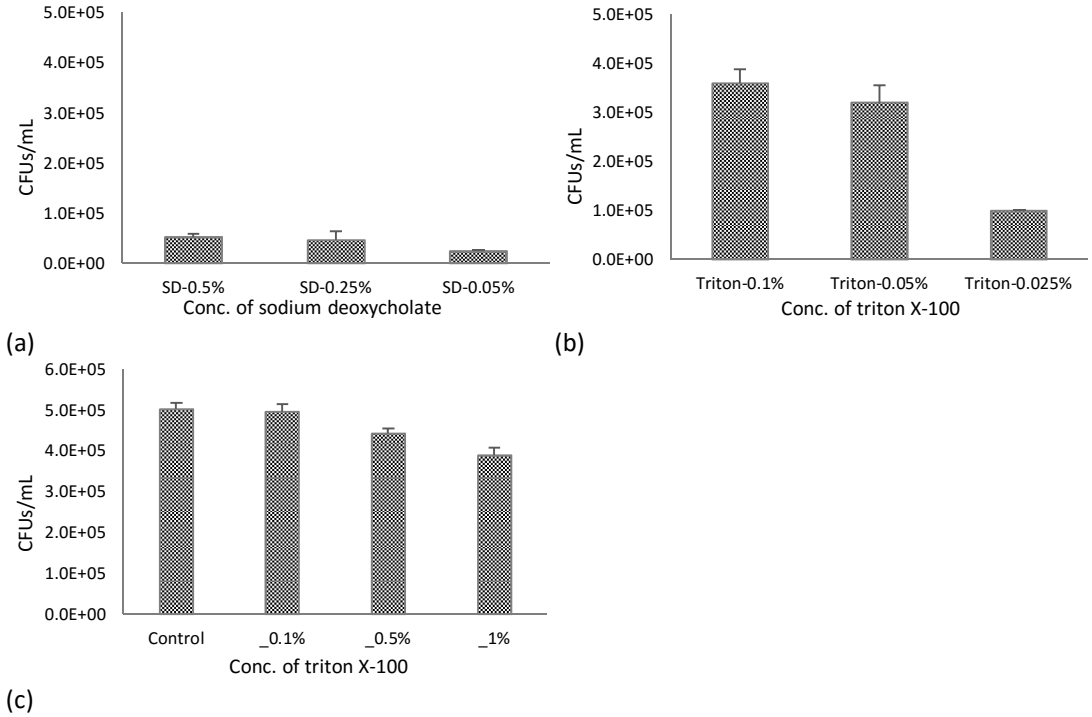


Figure 3.9: The effect of detergents on the lysis of *Acanthamoeba* cells using sodium deoxycholate (a) and triton-X100 (b) and the viability of recovered *A. butzleri* from infected cells by use of triton. Bacteria were exposed to different concentrations of triton for 5 min and then serially diluted before plating them onto the VD agar plates for the colony counts.

3.3.2.3. Efficacy of gentamicin against *A. butzleri*

Gentamicin had very good efficacy for killing *A. butzleri* in 1h at 200 µg/mL (Figure 3.10). No growth was observed for gentamicin treated bacteria indicating that it is very effective at killing *A. butzleri* in 1h. Furthermore, no effect of gentamicin at this concentration was observed on the morphology or encystation of *Acanthamoeba* Neff.

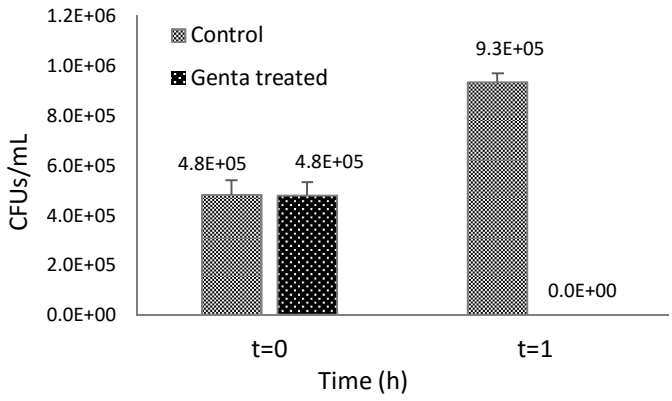


Figure 3.10: The effect of gentamicin (200 µg/mL) on *A. butzleri*. The bacterial culture was exposed to gentamicin for 1h followed by serial dilutions and plating on VD agar plates. Gentamicin killed the bacteria completely. The data represents mean±SE of three independent experiments.

3.3.3. Uptake of *A. butzleri* by *Acanthamoeba*

The first step in the interaction of *Acanthamoeba* and bacteria is attachment followed by entry into the cells. These processes were studied in detail as described below.

3.3.3.1. Attachment of *A. butzleri* with *Acanthamoeba* by "capping"

Visual inspection of the interaction between *A. butzleri* (ED-1) and *A. castellanii* Neff was studied to find how these organisms behave when they are brought together in the same medium. After the infection, motile bacteria could be seen moving around and trying to attach to the *Acanthamoeba* cell surface. The bacteria could be seen sticking to the outer surfaces of the cells all around and beating vigorously as if attempting to pierce through the cell membrane the interesting finding was that they concentrate at a single point eventually leading to the formation of a cap-like structure on the surface of the cell (Figure 3.11-a,b). The phenomenon of "capping" between *A. butzleri* and *Acanthamoeba* was also confirmed by using the fluorescent *A. butzleri* bacteria (ED-1+pJK1 expressing GFP) (Figure 3.11-c). Only one cap was seen per cell.

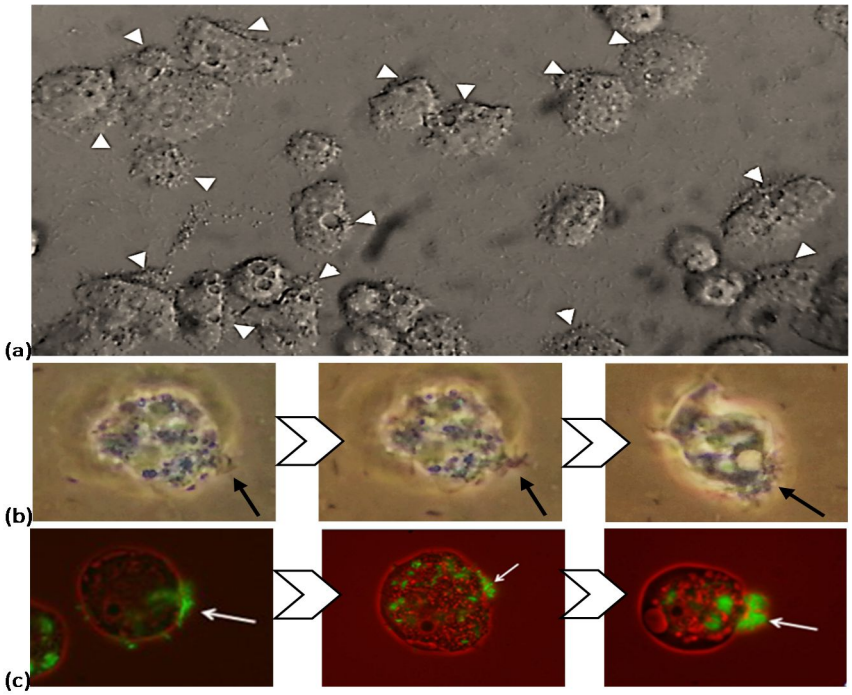


Figure 3.11: The "capping" mode of infection of *A. castellanii* Neff *Acanthamoeba* with *A. butzleri* ED-1. (a) Wider view of cells infected with *A. butzleri* ED-1 showing bacteria attached to the surface of the amoeba cells in various stages of capping (arrow heads). (b) The sequence of *A. butzleri* cap formation on *Acanthamoeba* cell; arrows show the location of cap formation. (c) The capping sequence of *A. butzleri* (ED-1+pJK1) on the surface of *Acanthamoeba* cell under fluorescence microscope; arrows indicate cap location.

The process of capping in *A. butzleri* is yet to be fully investigated but based on the observations it is probably linked to the changes in the cell surface after initial attachment of the *A. butzleri* cells that prevent any further capping on the cell. Capping does not seem to be the first step in uptake of *A. butzleri* into *Acanthamoeba* cell as the bacteria can be seen inside *Acanthamoeba* in vacuole even before the cap is fully formed which indicates continuous uptake of *A. butzleri* during the formation of the cap. The number of *A. butzleri* engulfed by *Acanthamoeba* (as observed in the vacuoles) seems to be related to the capping phenomenon. The *Acanthamoeba* cells with capping of *A. butzleri* appeared to have greater numbers of *A. butzleri* as compared to the cells where no capping was seen. The important thing is that there was wide variation among the *Acanthamoeba* cell population in the way they interact with *A. butzleri* as capping was not seen in all the cells.

3.3.3.2. Engulfment of *A. butzleri* by *Acanthamoeba*

Acanthamoeba Neff cells were seen to phagocytise *A. butzleri*. However, no evidence of coiling phagocytosis was found. Typical pseudopod formation was seen to accomplish the process (Figure 3.12).

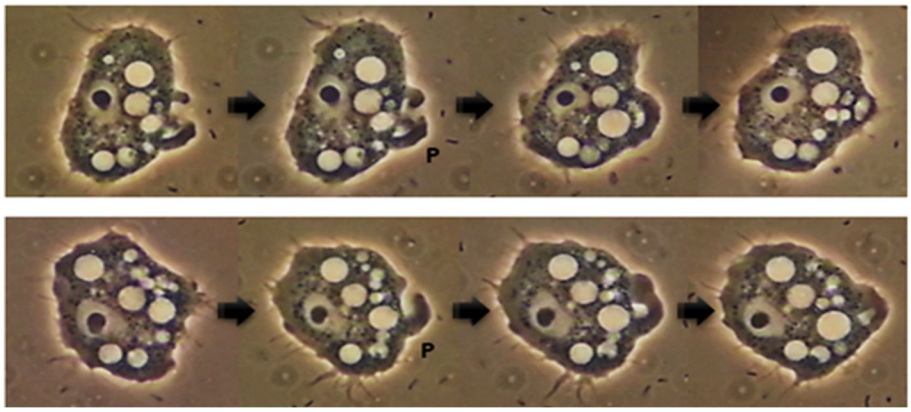
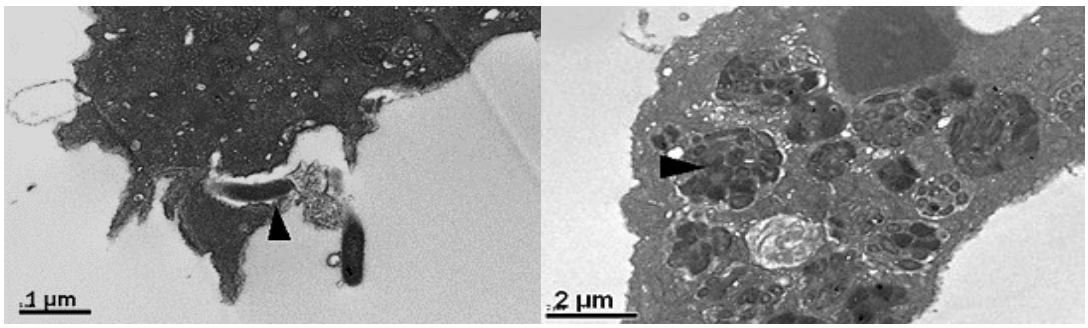
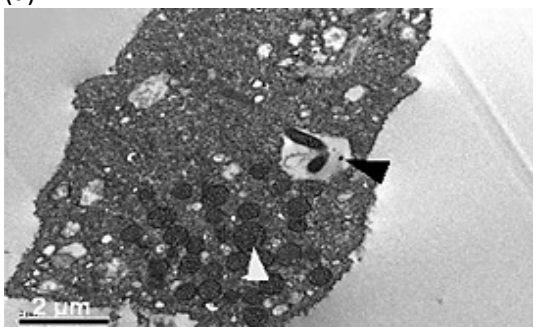


Figure 3.12: *Acanthamoeba castellanii* Neff phagocytising *A. butzleri* at two different instances (top and bottom rows). Typical pseudopodia (P) can be seen engulfing the bacteria on the surface of the cell.

Phagocytosis was also evident on electron micrographs as well (Figure 3.13), however, again no evidence of coiling phagocytosis was found. As with phagocytic cells, after phagocytosis by *Acanthamoeba*, *A. butzleri* could be seen inside the vacuoles. The presence of mitochondria in close proximity of vacuoles with internalized *A. butzleri* was also visible.



(a) (b)



(c)

Figure 3.13: Transmission electron micrograph of *A. castellanii* Neff infected with *A. butzleri* ED-1. (a) Typical pseudopods can be seen extending around and wrapping the bacterial cells. (b) A heavily infected Neff cell with *A. butzleri* ED-1. The bacteria (arrow-head) can be seen inside the vacuoles after being internalized. (c) Neff cell with internalized *A. butzleri* in vacuole (black arrow-head) while a large number of mitochondria (white arrow-head) can also be seen in close vicinity.

Using the fluorescent strain (ED-1_GFP) it could be seen that the capping phenomenon was visible as early as 15 min post-infection followed by appearance of motile bacteria inside vacuoles (arrows). Lysis of few cells was evident by 2.5h (Figure 3.14).

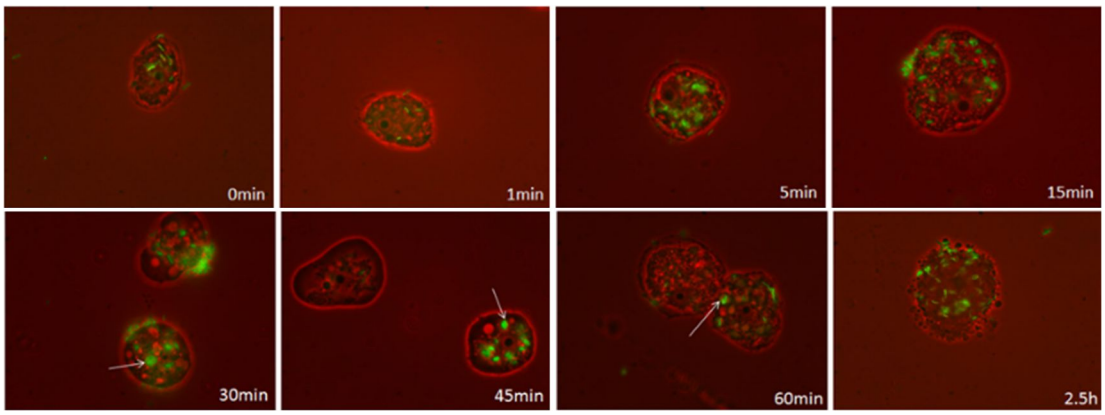


Figure 3.14: Time-course of infection captured with fluorescent microscope using *A. castellanii* Neff and ED-1_GFP. Photographs were taken at different time points. The capping bacteria could be seen as early as 15 min time point. Arrows indicate internalized bacteria. A lysed cell can be seen in the last time point.

3.3.3.3. Timescale of *Acanthamoeba* - *A. butzleri* interaction

After the infection of *Acanthamoeba* with *A. butzleri*, the bacteria could be seen to interact with the cells and attaching to the surface of the cells almost immediately while the bacteria could be seen within the vacuoles as early as 8-10 min. The process of capping could be seen between 10-45 min and by 1h bacteria almost don't seem to be interacting with the cells indicating that approximately 1h is possibly the optimum time for interaction of *A. butzleri* with *Acanthamoeba*. The evidence of lysis of *Acanthamoeba* cells can be seen as early as 45 min. However, no lysis is observed by 24h. The sequence of events that takes place after *Acanthamoeba* and *A. butzleri* come in contact with each other, is illustrated in Figure 3.15.

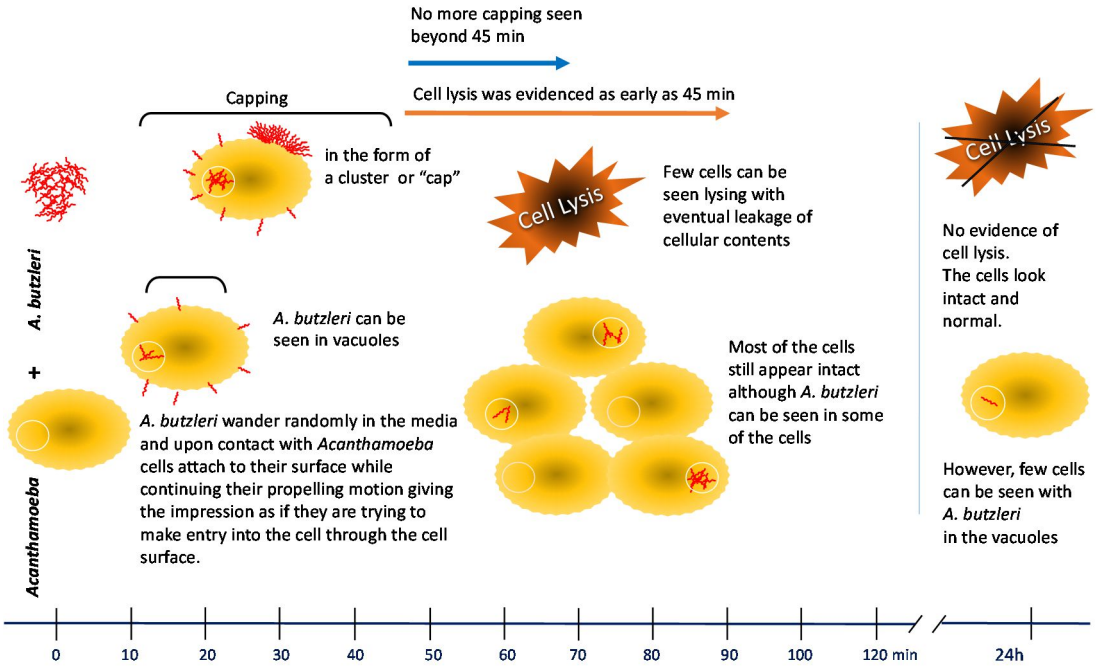


Figure 3.15: Diagrammatic representation of the sequence of the events that take place following interaction of *A. butzleri* with *A. castellanii* Neff. Soon after adding *A. butzleri* to *Acanthamoeba* cells, both start to interact with each other. The motile *A. butzleri* start to gather around the amoeba cells. Bacterial entry into the cells can be observed by 8-10 min while bacteria still keep piling up on the surface of the amoeba cell resulting in the formation of a cap-like structure. The capping phenomenon can be seen between 10-45 min. After that amoebal cells seem to lose their attraction for *A. butzleri*. By 45 min lysing cells can be seen. However, by 24h no lysis is observed and amoebal cells appear normal.

3.3.3.4. Role of sugars in phagocytosis of *A. butzleri* by *Acanthamoeba*

These experiments were aimed at studying the role of monosaccharide sugars (glucose, galactose and mannose) in attachment of *A. butzleri* strains (ED-1, Arco-L and RM-4018) with *Acanthamoeba* Neff and three soil isolates (Eco, Ent and Arc). The bacterial CFUs were counted at time zero of the infection. The non-treated controls were arbitrarily set to 100% and the values of treated groups were expressed as percent of control. Statistical difference of the effect of sugar treatment was determined by applying Student's t-test using GraphPad software.

Blocking of sugar receptors on cell membrane (sugar pre-treatment) significantly reduced the uptake of all the three types of the bacteria (ED-1, Arco-L and RM-4018) for either of the sugars (glucose, galactose and mannose) at 100 mM as well as 50 mM concentrations, while at 10 mM concentration the uptake varied among the sugars and the bacterial strains (Figure 3.16). In glucose pre-treatment group, ED-1 strain was least affected by increased concentration of glucose while in galactose group, RM-4018 was the least affected. Maximum effect was observed for mannose (100 mM) and ED-1 and Arco-L strains. These strains also had the least uptake in galactose and glucose groups as well. Glucose appeared to have the least effect on the uptake of bacteria. However, in glucose group at 100 mM concentration, RM-4018 strain had least uptake contrary to galactose and glucose groups.

Overall, the sugar pre-treatment manifested an effect on the uptake of the three *A. butzleri* strains by *Acanthamoeba* cells in a concentration-dependent manner although there were differences among these bacterial strains and among the three sugar groups. These results indicate the importance of monosaccharide sugars in the uptake of *A. butzleri* by *Acanthamoeba* Neff. Furthermore, ED-1 and Arco-L strains seem to utilize more the galactose and mannose channels while RM-4018 appears to rely more on glucose channels on the surface of *Acanthamoeba*.

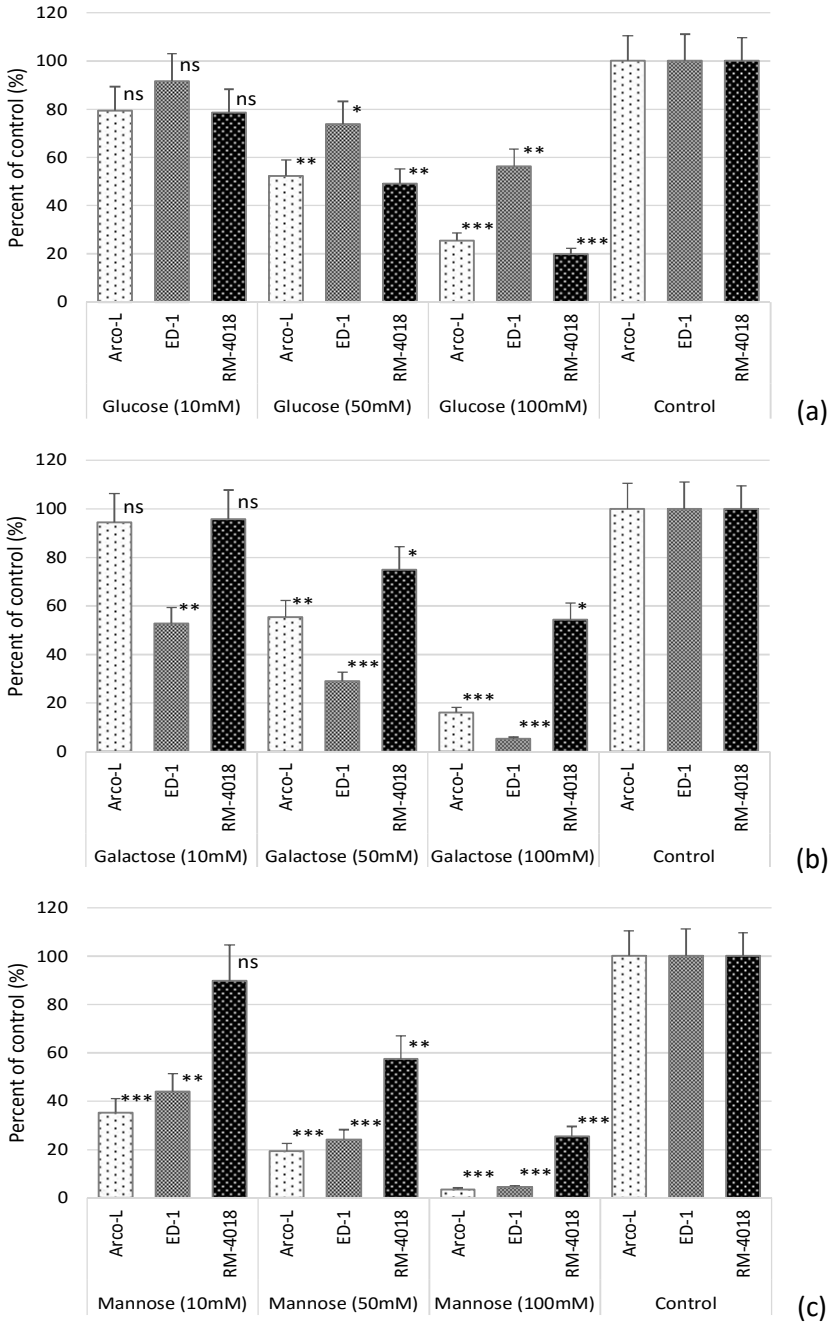


Figure 3.16: Effect of pre-treatment of glucose (a), galactose (b) and mannose (c) on the uptake of strains of *A. butzleri* (ED-1, Arco-L and RM-4018) by *A. castellanii* Neff. Washed amoeba cells were left in NS with various concentrations of these sugars (10, 50 and 100 mM) for 1h before infecting them with the *A. butzleri* strains (MOI=100:1) in gentamicin protection assay. Bacteria were recovered by cell lysis at t=0 and the CFUs were counted. The data was converted into percentage by comparing CFUs of treatment groups with the untreated control while the entry of each control group was arbitrarily set to 100%. The blocking effect of sugars on the uptake of *A. butzleri* strains was observed which indicates the role of sugar receptors on *Acanthamoeba* cell surface in interaction and internalization of these bacteria in a concentration-dependent manner. The data represents mean±SE of three independent experiments. An asterisk (*) represents significant difference compared to the respective control as determined by the Student's t-test using GraphPad software (*p≤0.05; **p≤0.01; ***p≤0.001; ns=non-significant).

The effect of sugars on the uptake of *A. butzleri* strains was also studied for three different soil isolates of *Acanthamoeba* other than Neff strain including one isolate each from *E. coli* (Eco), *Enterococcus* (Ent) and *Arcobacter* (Arc) which all belonged to T4 genotype although different subgroups (i.e. T4-A, T4-B and T4-E respectively) (Section 3.2.6.). The results were quite varied among the three isolates of *Acanthamoeba* as well as *A. butzleri* (Figure 3.17) although the effect was concentration-dependent in all the cases. .

The Eco isolate appeared to be the most vulnerable to the effect of sugars especially to mannose treatment for the uptake of *A. butzleri*. Moreover, RM-4018 strain did not seem to be affected much in Ent isolate of *Acanthamoeba*. These results show variations in the effect of sugars on the uptake of various strains of *A. butzleri* although almost all of them are affected by the sugar pre-treatment. Therefore, presence of sugar receptors on the surface of *Acanthamoeba* cells and their primary involvement in the uptake of *A. butzleri* seem quite obvious.

Among the glucose treatment group, the maximum effect was observed with Arco-L strain of *A. butzleri* and Arc isolate of *Acanthamoeba*. In galactose group, the maximum effect was seen for ED-1 and Eco combination while in mannose group the blocking effect was greatest for Arco-L/ED-1 and Eco isolate. The wide variations in the pattern of blockage of bacteria by different strains of *Acanthamoeba* indicates that although all the three types of sugars (glucose, galactose and mannose) seem to play role in uptake of *A. butzleri* but the effect is not uniform .

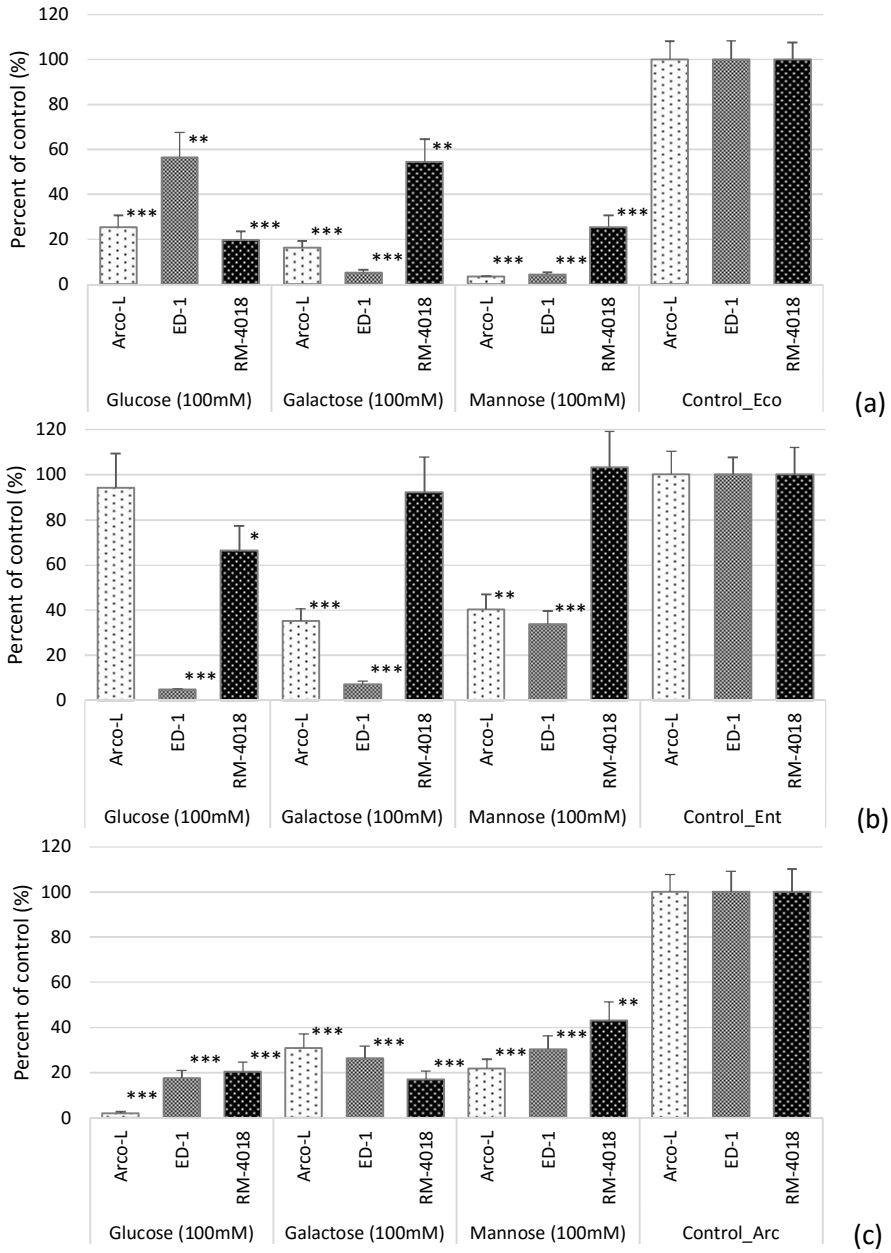


Figure 3.17: Effect of glucose, galactose and mannose pre-treatment on the uptake of three different strains of *A. butzleri* (ED-1, Arco-L and RM-4018) by three different *Acanthamoeba*; **Eco** isolate (a), **Ent** isolate (b) and **Arc** isolate (c). Washed *Acanthamoeba* cells were left in NS with various concentrations of glucose (10, 50 and 100 mM) for 1h before infecting them with the *A. butzleri* strains (MOI=100:1) in gentamicin protection assay. Bacteria were recovered by cell lysis at t=0 and the CFUs were counted. The data was converted into percentage by comparing CFUs of treatment groups with the untreated control while the entry of each control group was arbitrarily set to 100%. There are wide variations in the pattern of blockage of bacteria by different strains of *Acanthamoeba*. This indicates that although all the three types of sugars used seem to play their role in uptake of *A. butzleri* but the effect is not uniform and probably indicates uneven distribution of these receptors on the surface of various *Acanthamoeba* isolates which might be responsible for different uptakes of bacteria by different isolates. The data represents mean±SE of three independent experiments. An asterisk (*) represents significant difference compared to the respective control as determined by the Student's t-test using GraphPad software (*p≤0.05; **p≤0.01; ***p≤0.001; ns=non-significant).

3.3.3.5. Role of phagocytosis inhibitors in uptake of *A. butzleri* by *Acanthamoeba*

To understand whether *Acanthamoeba* use similar strategies and pathways as commonly used by other phagocytic cells, a number of inhibitors were used.

3.3.3.5.1. Role of actin polymerization in uptake of *A. butzleri*

Actin polymerization is an important feature which is required, among many other activities, for the movement of the *Acanthamoeba* cell membrane and thereby for pseudopods formation. Cytochalasin D is an actin polymerization inhibitor which reduced the uptake of *A. butzleri* by *Acanthamoeba* significantly for all the three bacterial strains used in a concentration-dependent manner (Figure 3.18). These results indicate that cytochalasin D affects actin polymerisation within *Acanthamoeba* trophozoite cells to such an extent that cytoskeletal rearrangement necessary for the phagocytosis of *A. butzleri* is halted. Therefore, tyrosine kinase-mediated signalling of actin polymerisation plays a vital role in the phagocytosis of *A. butzleri* by *Acanthamoeba.*

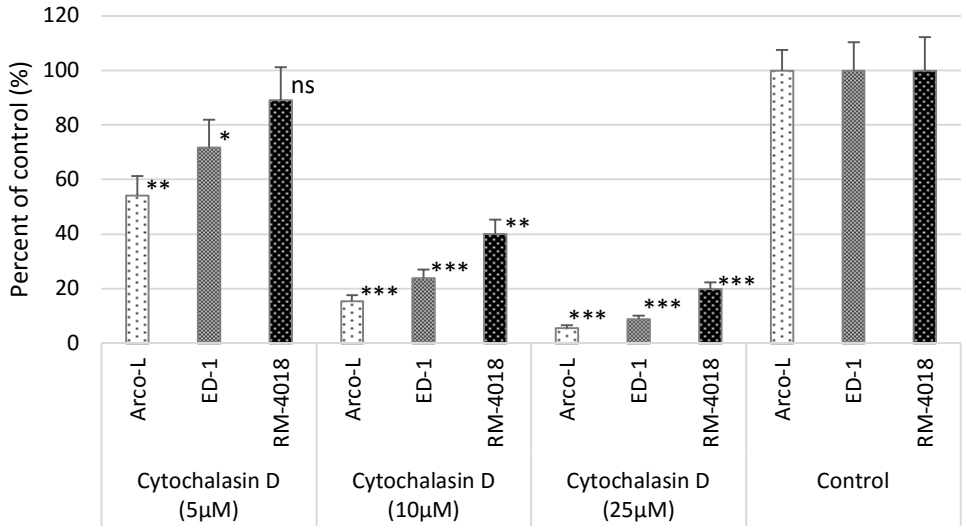


Figure 3.18: The effect of pre-treatment of cytochalasin D on the uptake of three different strains of *A. butzleri* (ED-1, Arco-L and RM-4018) by *Acanthamoeba* Neff. Washed *Acanthamoeba* Neff cells were left in NS with various concentrations of cytochalasin D (5, 10 and 25 100 µM) for 1h before infecting them with the *A. butzleri* strains (MOI=100:1) in gentamicin protection assay. Bacteria were recovered by cell lysis at t=0 and the CFUs were counted. The data was converted into percentage by comparing CFUs of treatment groups with the untreated control while the entry of each control group was arbitrarily set to 100%. The data represents mean±SE of three independent experiments. An asterisk (*) represents significant difference compared to the respective control as determined by the Student's t-test using GraphPad software (*p≤0.05; **p≤0.01; ***p≤0.001; ns=non-significant).

3.3.3.5.2. Role of PI3-K signalling pathway in uptake of *A. butzleri*

Pre-treatment of *Acanthamoeba* cells with various concentrations of wortmannin caused a sharp drop in uptake of all the three strains of *A. butzleri* (Figure 3.19). The effect was concentration-dependent and was most evident at 1000 nM while ED-1 and Arco-L strains of *A. butzleri* appeared to be most affected. This reinforces the findings of the actin polymerization inhibition by cytochalasin D that actin polymerization plays an important part in the uptake of *A. butzleri* by *Acanthamoeba*.

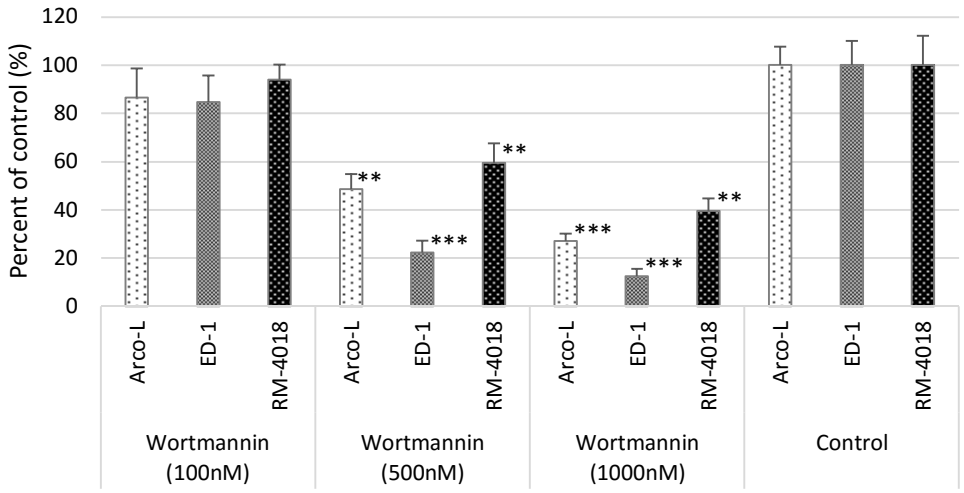


Figure 3.19: The effect of pre-treatment of wortmannin on the uptake of three different strains of *A. butzleri* (ED-1, Arco-L and RM-4018) by *Acanthamoeba* Neff. Washed *Acanthamoeba* Neff cells were left in NS with various concentrations of wortmannin (100, 500 and 1000 nM) for 1h before infecting them with the *A. butzleri* strains (MOI=100:1) in gentamicin protection assay. Bacteria were recovered by cell lysis at t=0 and the CFUs were counted. The data was converted into percentage by comparing CFUs of treatment groups with the untreated control while the entry of each control group was arbitrarily set to 100%. The data represents mean±SE of three independent experiments. An asterisk (*) represents significant difference compared to the respective control as determined by the Student's t-test using GraphPad software (*p≤0.05; **p≤0.01; ***p≤0.001; ns=non-significant).

3.3.3.5.3. Role of protein tyrosine phosphatase (PTP) inhibitors

SoV which is specifically an inhibitor of PTP was used to disrupt the intracellular signalling pathway. However, this resulted in increased uptake of bacteria (Figure 3.20). The effect was concentration-dependent and was most prominent in RM-4018 strain. Heavily infected cells with abundant motile *A. butzleri* in vacuoles could be seen in infected *Acanthamoeba* cells (Figure 3.21). These findings indicate that phagocytosis of *A. butzleri* by *Acanthamoeba* is dependent upon intracellular signalling pathways.

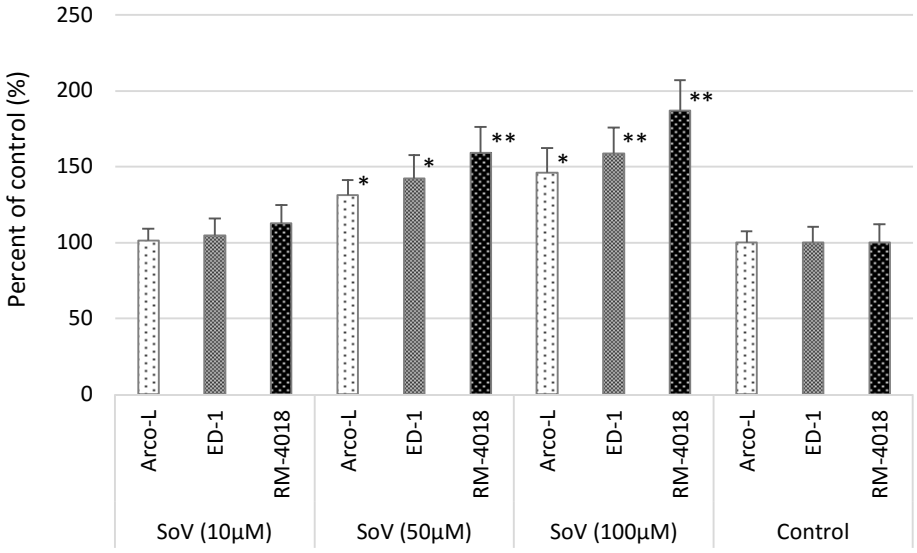


Figure 3.20: The effect of pre-treatment of sodium orthovanadate on the uptake of three different strains of *A. butzleri* (ED-1, Arco-L and RM-4018) by *Acanthamoeba* Neff. Washed *Acanthamoeba* Neff cells were left in NS with various concentrations of sodium orthovanadate (10, 50 and 100 µM) for 1h before infecting them with the *A. butzleri* strains (MOI=100:1) in gentamicin protection assay. Bacteria were recovered by cell lysis at t=0 and the CFUs were counted. The data was converted into percentage by comparing CFUs of treatment groups with the untreated control while the entry of each control group was arbitrarily set to 100%. The data represents mean±SE of three independent experiments. An asterisk (*) represents significant difference compared to the respective control as determined by the Student's t-test using GraphPad software (*p≤0.05; **p≤0.01; ***p≤0.001; ns=non-significant).

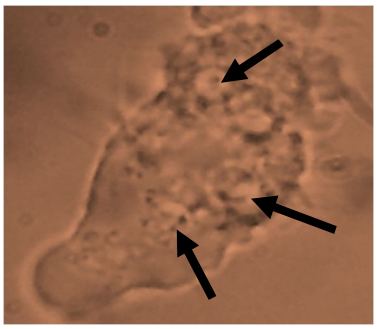


Figure 3.21: *A. castellanii* Neff cells infected by *A. butzleri* after 1h treatment with sodium orthovanadate. Heavily infected vacuoles containing large number of bacteria can be seen (arrows).

3.3.3.5.4. Effect of neutralization of acidification and enhanced pH on the survival of *A. butzleri* in phagolysosome

The effect of both the neutralization of acidification within the phagolysosome as well as the effect of enhanced pH on the survival of *A. butzleri* in phagolysosome of

Acanthamoeba was assessed by the use of ammonium chloride and monensisn treatments, respectively. Ammonium chloride is a weak base, which accumulates in the intracellular acidic compartments and neutralizes the pH. The addition of ammonium chloride did affect the killing of bacteria as reflected by the increases number of *A. butzleri* recovered from *Acanthamoeba* cells pre-treated with this base (Figure 3.22). The effect was concentration dependent.

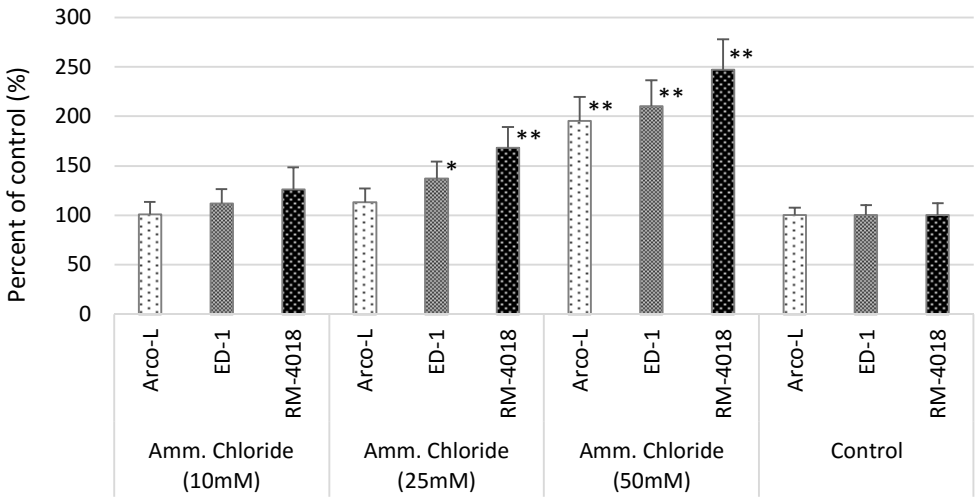


Figure 3.22: The effect of pre-treatment of ammonium chloride on the uptake of three different strains of *A. butzleri* (ED-1, Arco-L and RM-4018) by *Acanthamoeba* Neff. Washed *Acanthamoeba* cells were left in NS with various concentrations of ammonium chloride (10, 25 and 50 µM) for 1h before infecting them with the *A. butzleri* strains (MOI=100:1) in gentamicin protection assay. Bacteria were recovered by cell lysis at t=0 and the CFUs were counted. The data was converted into percentage by comparing CFUs of treatment groups with the untreated control while the entry of each control group was arbitrarily set to 100%. The data represents mean±SE of three independent experiments. An asterisk (*) represents significant difference compared to the respective control as determined by the Student's t-test using GraphPad software (*p≤0.05; **p≤0.01; ***p≤0.001; ns=non-significant).

The use of monensin before infection of *Acanthamoeba* with *A. butzleri* caused a drop in the uptake of the bacteria especially at the highest dosages (25 µM) (Figure 3.23). This indicates the role of intracellular transport system on the uptake of *A. butzleri* as well as the contribution of lysosomal pH in the killing of these bacteria.

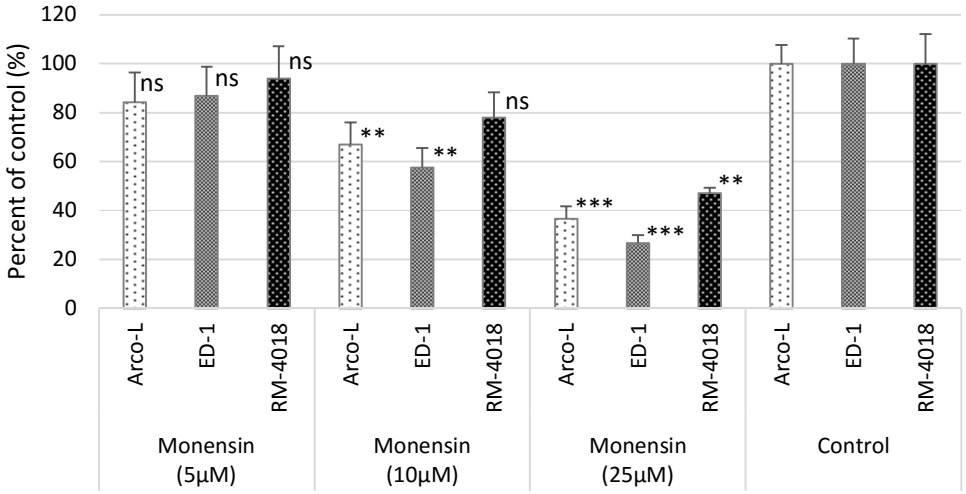


Figure 3.23: The effect of pre-treatment of monensin on the uptake of three different strains of *A. butzleri* (ED-1, Arco-L and RM-4018) by *Acanthamoeba* Neff. Washed *Acanthamoeba* cells were left in NS with various concentrations of monensin (5, 10 and 25 µM) for 1h before infecting them with the *A. butzleri* strains (MOI=100:1) in gentamicin protection assay. Bacteria were recovered by cell lysis at t=0 and the CFUs were counted. The data was converted into percentage by comparing CFUs of treatment groups with the untreated control while the entry of each control group was arbitrarily set to 100%. The data represents mean±SE of three independent experiments. An asterisk (*) represents significant difference compared to the respective control as determined by the Student's t-test using GraphPad software (*p≤0.05; **p≤0.01; ***p≤0.001; ns=non-significant).

3.3.3.5.5. Role of v-ATPases in intracellular survival of *A. butzleri*

Bafilomycin A blocks the v-ATPase system by attaching to its intra-membrane located V0 domain's subunit c. The pre-treatment of *Acanthamoeba* cells with bafilomycin A caused enhanced uptake and survival of *A. butzleri* (Figure 3.24 and 3.25). This provides a direct evidence of the role of v-ATPases as part of the killing mechanisms of *A. butzleri* by *Acanthamoeba*.

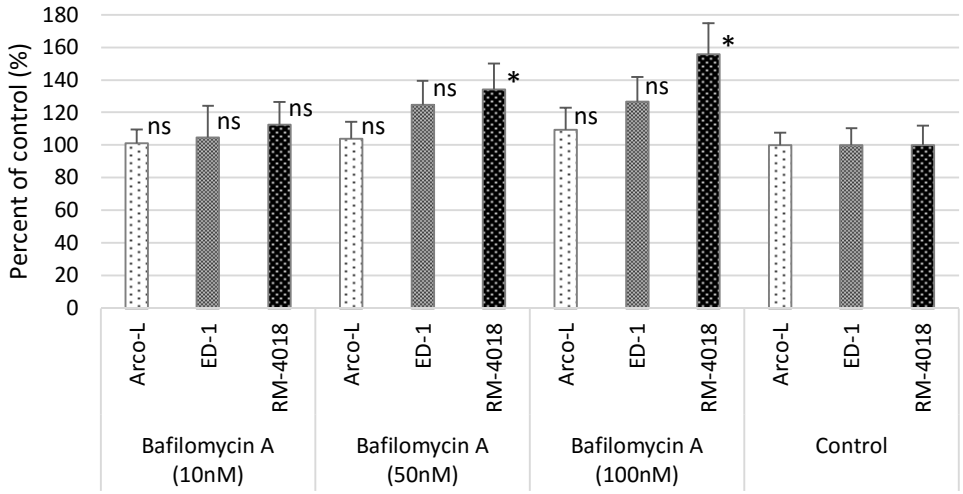


Figure 3.24: The effect of pre-treatment of bafilomycin A on the uptake of three different strains of *A. butzleri* (ED-1, Arco-L and RM-4018) by *Acanthamoeba* Neff. Washed *Acanthamoeba* cells were left in NS with various concentrations of Bafilomycin A (10, 50 and 100 µM) for 1h before infecting them with the *A. butzleri* strains (MOI=100:1) in gentamicin protection assay. Bacteria were recovered by cell lysis at t=0 and the CFUs were counted. The data was converted into percentage by comparing CFUs of treatment groups with the untreated control while the entry of each control group was arbitrarily set to 100%. The data represents mean±SE of three independent experiments. An asterisk (*) represents significant difference compared to the respective control as determined by the Student's t-test using GraphPad software (*p≤0.05; **p≤0.01; ***p≤0.001; ns=non-significant).

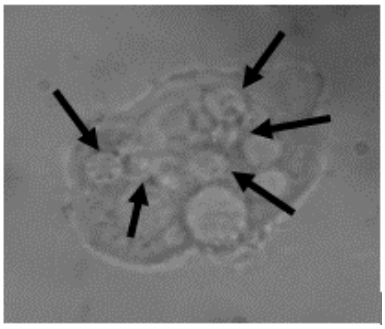


Figure 3.25: The effect of bafilomycin A pre-treatment on the uptake of *A. butzleri*. Many bacteria filled phagosomes can be seen (arrows) in the *Acanthamoeba* cell infected with *A. butzleri* following bafilomycin A exposure for 1h.

3.3.3.5.6. Mechanisms involved in killing of *A. butzleri* in phagolysosome of *Acanthamoeba*

After the fusion of lysosomes with the phagosome containing bacteria, the lysosomal enzymes are activated. All bacteria cannot resist this hostile environment and some escape the phagolysosome into the cytoplasm and survive/proliferate there. However,

this does not seem to be a case with *A. butzleri*. It seems to resist the lysosomal enzyme action to a certain extent (Section 3.3.4.5. ; Figure 3.33). The number of *A. butzleri* bacteria in *Acanthamoeba* cells treated with suramin was higher than the non-treated cells indicating the direct involvement of lysosomal enzymes in the killing of *A. butzleri* strains used (Figure 3.26). This also confirms that these bacteria do not escape the phagosome and are retained there.

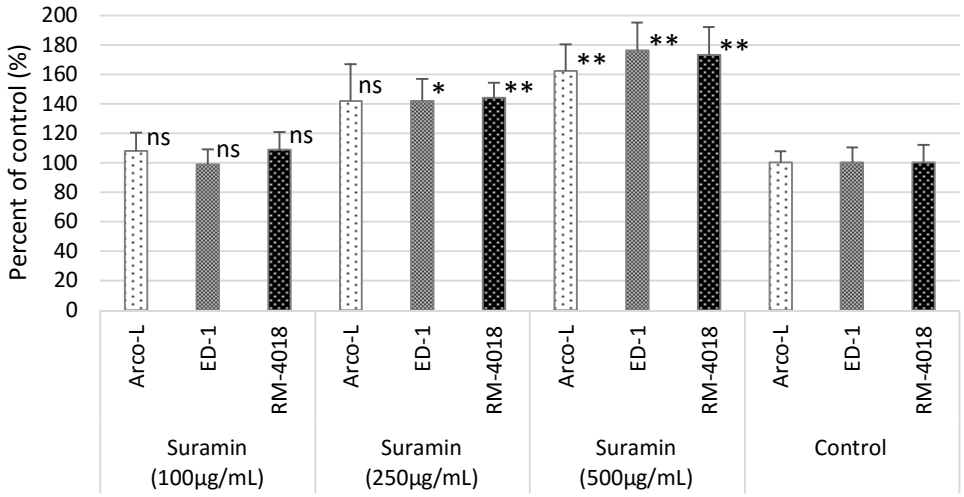


Figure 3.26: The effect of pre-treatment of suramin on the uptake of three different strains of *A. butzleri* (ED-1, Arco-L and RM-4018) by *Acanthamoeba* Neff. Washed *Acanthamoeba* cells were left in NS with various concentrations of suramin (100, 250 and 500 µg/mL) for 1h before infecting them with the *A. butzleri* strains (MOI=100:1) in gentamicin protection assay. Bacteria were recovered by cell lysis at t=0 and the CFUs were counted. The data was converted into percentage by comparing CFUs of treatment groups with the untreated control while the entry of each control group was arbitrarily set to 100%. The data represents mean±SE of three independent experiments. An asterisk (*) represents significant difference compared to the respective control as determined by the Student's t-test using GraphPad software (*p≤0.05; **p≤0.01; ***p≤0.001; ns=non-significant).

Compiling all the results from the experiments with sugar pre-treatment, phagocytosis inhibitors and phagosomal acidification inhibitors, the net picture that emerges of the mechanisms involved in the process of phagocytosis of *A. butzleri* by *Acanthamoeba* is drawn in Figure 3.27.

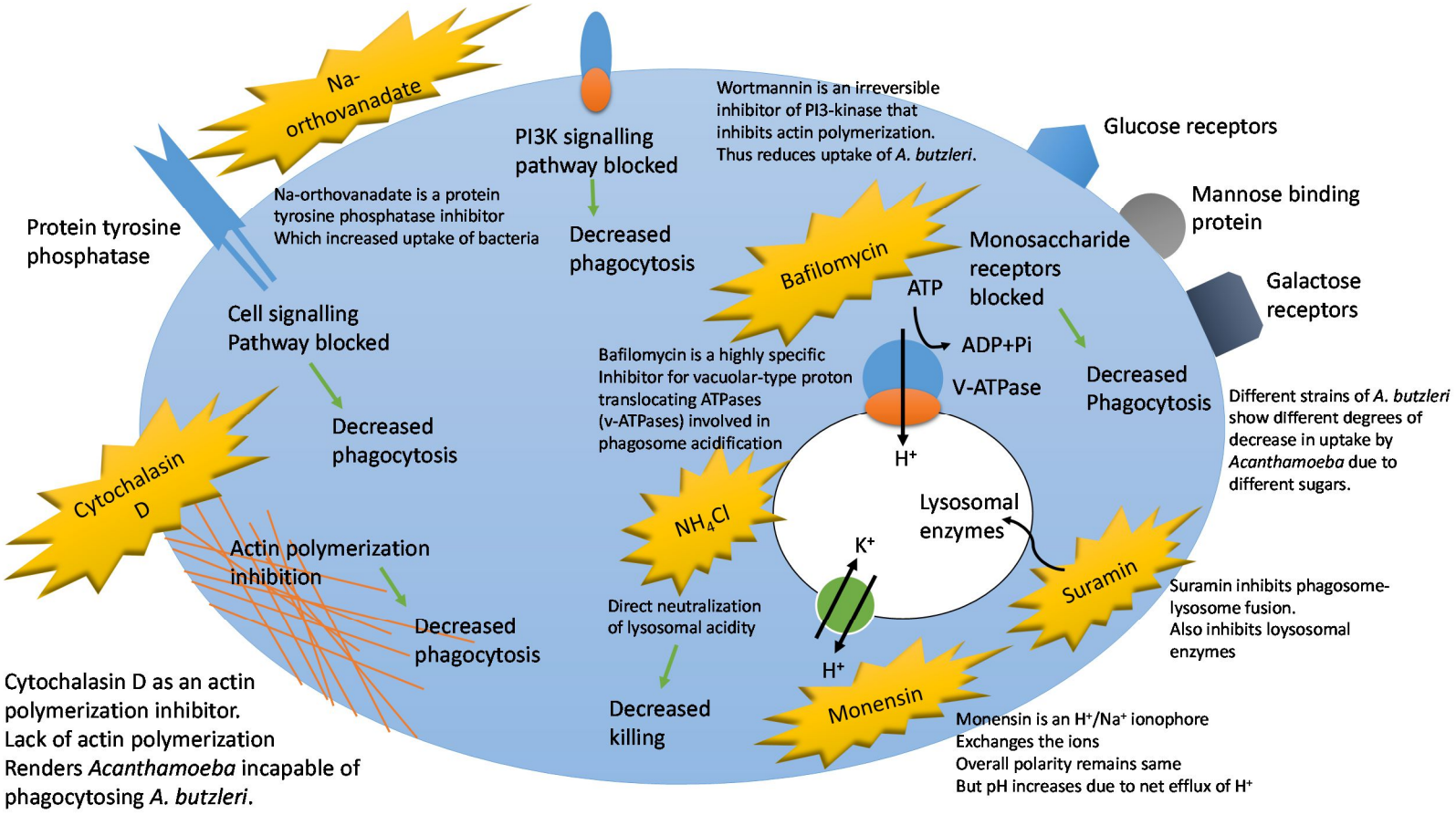


Figure 3.27: The various cellular and molecular mechanisms involved in the entry and killing of *A. butzleri* in *Acanthamoeba* based mainly on observations in Neff strain. These were determined by the inhibitory studies blocking various pathways to determine the role of each pathway in the interaction of this host-predator pair. These included use of sugars (**glucose, galactose, mannose**); actin polymerization inhibitor (**cytochalasin D**); PI3K inhibitor (**wortmannin**); tyrosine protein phosphatase inhibitor (**sodium orthovanadate**); a weak base (**ammonium chloride**); an H^+/Na^+ ionophore (**monensin**); inhibitor of lysosome-phagosome fusion (**suramin**); an inhibitor for v-ATPases (**bafilomycin A**). The pre-treatment was followed by washing and infection with *A. butzleri* strains.

3.3.4. Intracellular survival of *A. butzleri* in *Acanthamoeba* (infection assays)

It was observed that after co-incubating *Acanthamoeba* and *A. butzleri*, all the cells are not infected, rather 60% cells were found to be infected and having motile bacteria inside the vacuoles (Figure 3.28), while 36.2% were uninfected and 3.8% had lysed.

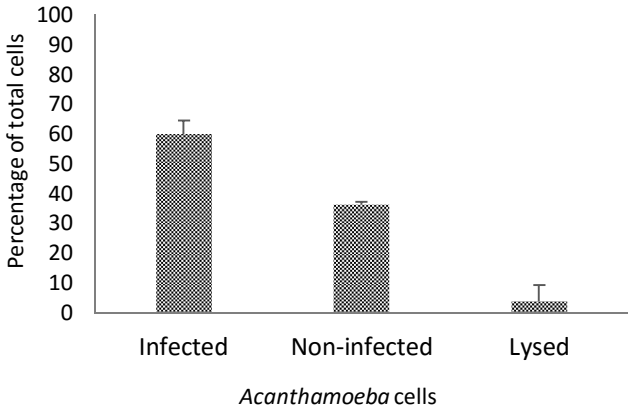


Figure 3.28: The comparative proportion of infected, non-infected and lysed cells when *A. castellanii* Neff are infected with *A. butzleri* ED-1 at an MOI of 100:1. The observations were made 1h post-infection. The data represents mean±SE of three experiments.

3.3.4.1. Lysis of *Acanthamoeba* cells after infection with *A. butzleri*

To further confirm the lysis of *Acanthamoeba* cells by *A. butzleri* a simple experiment was setup in a 24-well plate with a monolayer of *A. castellanii* Neff. Except control, all the monolayers were infected with *A. butzleri* (ED-1) for 1h at an MOI of 100:1. After that the first well was washed with saline, in the second well only supernatant was removed (but no washing) while the third well as well as the uninfected control well were left as such with no change. The cells in all the wells were stained by trypan blue and observed under microscope for any lysis of cells. The results are shown in Figure 3.29. It is clear from the results that, firstly, the infection of *Acanthamoeba* cells with *A. butzleri* causes the cells to detach more from the surface of the plate as compared to the uninfected control. Secondly, it also shows that some of the cells are lysed which can easily be seen in Figure 3.29d.

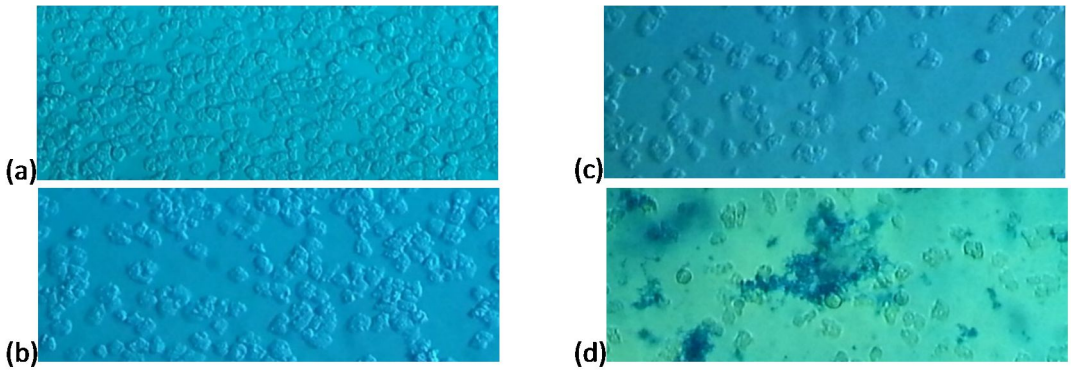


Figure 3.29: Trypan blue staining of cells for evidence of lysis when *A. castellanii* Neff are infected with *A. butzleri* ED-1 (MOI=100). The cell monolayers in 24-well plate were infected with bacteria for 1h and then stained with trypan blue for any lysed/dead cells. (a) Uninfected control cells. (b) Infected cells; the supernatant was removed after infection and the monolayer was washed with Neff's saline before staining. (c) infected cells; only the supernatant was removed but cells were not washed before staining. (d) infected cells; the supernatant was not removed so this well has all the cells whether alive or dead. The dead/lysed cells can be seen aggregating. It is also clear that the infected cells are more likely to detach from the surface of the plate as compared to the non-infected cells.

3.3.4.2. The lysis of *Acanthamoeba* cells is caused by *A. butzleri* and not the secretory factors

The effect of *A. butzleri*-grown media in parallel with *A. butzleri* on the lysis of *Acanthamoeba* cells was assessed. After 1h of incubation with *A. butzleri*, some of the infected cells could be seen lysed but no cell was lysed in the well with *A. butzleri*-grown media even after 72h incubation. This confirms that the lysis of *Acanthamoeba* cells by *A. butzleri* is directly related to the invasion of bacteria and not because of any factor released by the bacteria in the medium.

3.3.4.3. *Acanthamoeba* cell lysis sequence

The lysis of *Acanthamoeba* cell following infection with *A. butzleri* was found to be accomplished under a specific pattern that included a number of changes. The cell became less mobilized and very few locomotive pseudopodia could be seen. Usually a heavily infected cell was seen lysing which was accompanied by presence of a number of vacuoles in the cell. Bacteria inside the vacuoles were clearly visible with their typical beating movements. The cell membrane ruptured at a specific point with the release of the cellular content. The cellular organelles and the compartments also started to degrade including the nucleus and the vacuoles with the release of bacteria and complete degradation of the cell (Figure 3.30).

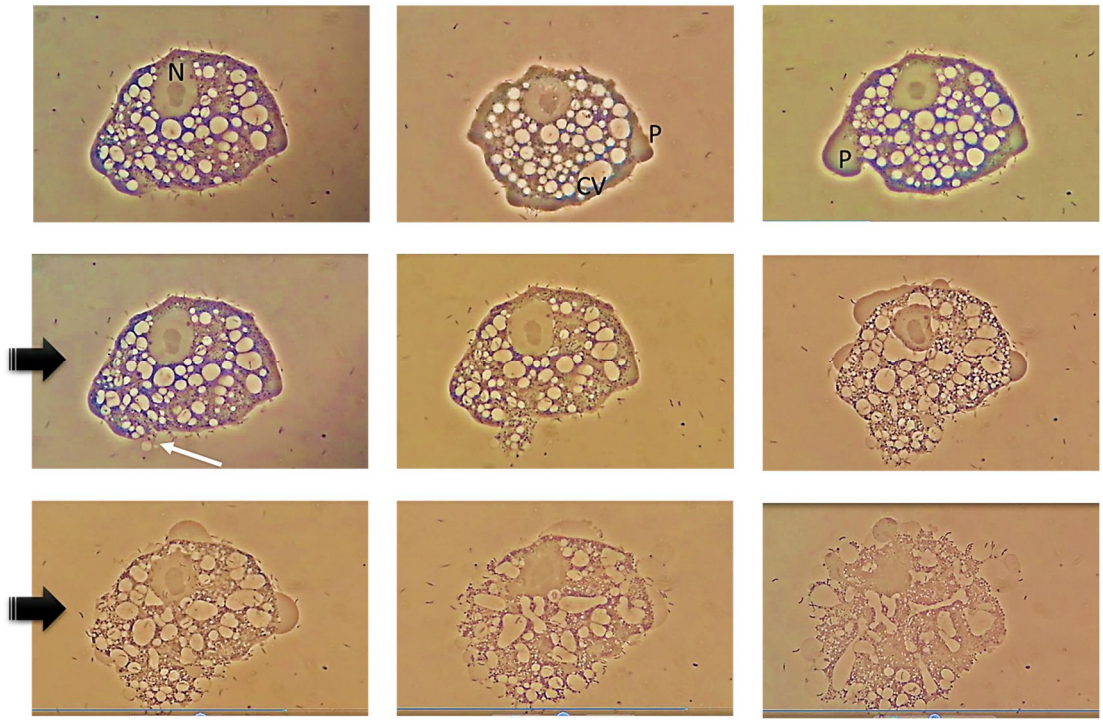


Figure 3.30: Time lapse photography *A. castellanii* Neff cell undergoing lysis after infection with *A. butzleri* ED-1 (sequenced from top left to bottom right). Numerous vacuoles can be seen in the cell which has been observed in other cells as well undergoing lysis. Other normal structures including nucleus (N), contractile vacuole (CV) and pseudopods (P) can also be seen before lysis. Although the cell becomes sluggish, immobile and nearly round, the formation of pseudopods continues until quite late. Eventually the cell membrane ruptures at a point with the leakage of the cell content. Later the cell completely disintegrates including the cell organelles.

A better view of the events and changes in the lysed cells could be seen at higher magnification and further in electron microscope. Large number of vacuoles with bacteria could be seen inside the cells before lysis (Figure 3.31a). Lysis resulted in massive destruction of the cellular structures including nucleus and phagosomes containing bacteria. The released bacteria were still alive as manifested by their beating movements (Figure 3.31b). These findings were further consolidated by viewing lysed cells under transmission electron microscope (Figure 3.31c).

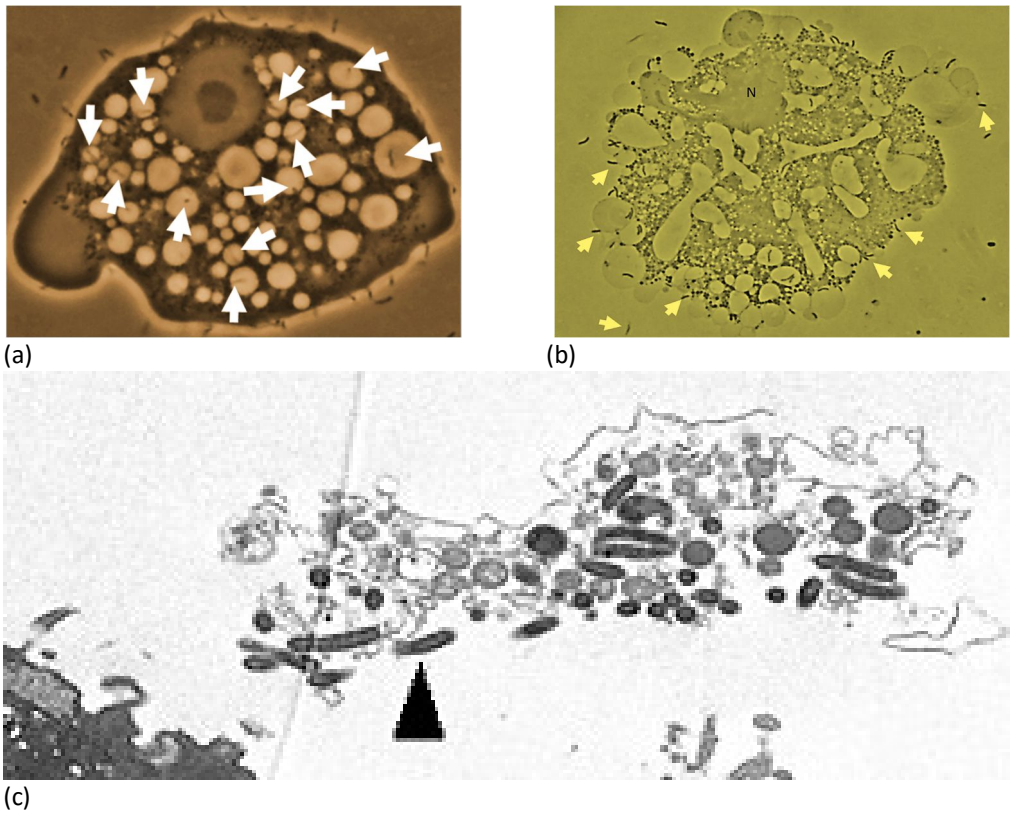


Figure 3.31: Lysis of *A. castellanii* Neff cell infected with *A. butzleri* ED-1. (a) Enlarged view of Neff cell before rupturing. A number of vacuoles can be seen inside the cells with the bacteria in them. Because of their constant beating movements some of the bacteria may not be visible in this view. (b) Enlarged view of Neff cells lysed due to infection viewed under light microscope. Lysis results in loss of integrity of the cellular organelles and compartments including nucleus (N). The rupture of phagosomes containing internalized bacteria results in release of these bacteria into the surrounding (arrows). (c) Transmission electron micrograph of a lysed Neff cell after infection. Numerous bacteria can be seen in the ruptured cell (arrow-head).

3.3.4.4. Internalization of *A. butzleri*

Internalization of *A. butzleri* strains by *A. castellanii* Neff indicated variations among the three strains to be taken up by the amoebal cells. Internalization was the greatest for ED-1 followed by Arco-L and RM-4018 (Figure 3.32).

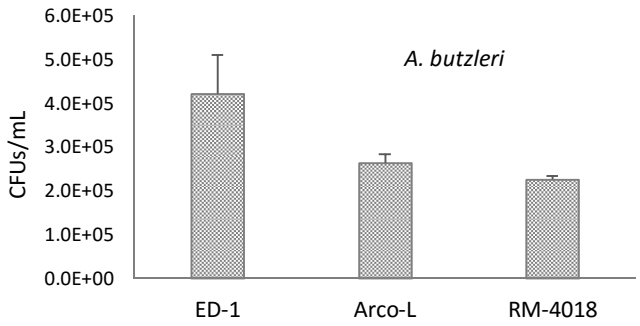


Figure 3.32: Comparison of internalization of *A. butzleri* strains ED-1, Arco-L and RM-4018 by *A. castellanii* Neff. The data represents mean±SE of three independent experiments.

3.3.4.5. Intracellular survival of *A. butzleri* ED-1 in *Acanthamoeba*

A. butzleri ED-1 had an internalization rate of 1.1% when infected at an MOI of 100:1. This means 1.1% of the total number of bacteria added to the *Acanthamoeba* cells suspension were ingested and managed to survive at time zero post-infection. The bacteria demonstrated a very interesting intracellular survival pattern; they proliferated for a short time (2h) after t=0 and lysed some *Acanthamoeba* cells as well, however, they started to decline afterwards and by 4h the number had declined to less than half of their initial number. This decline continued until 24h and then further by 48h (Figure 3.33). This shows that the bacteria are very proactive in the early stages of the infection but they cannot sustain it for longer and are dominated by hostile intracellular environment of *Acanthamoeba* which does not allow further proliferation but survival.

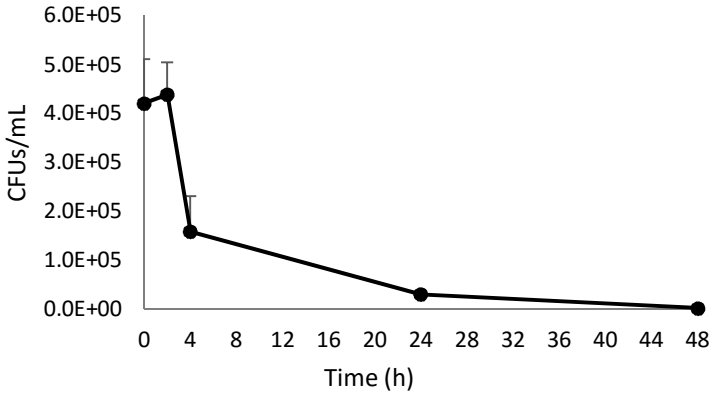


Figure 3.33: Intracellular survival pattern of *A. butzleri* ED-1 in *A. castellanii* Neff over a period of 48h. The data represents mean±SE of nine independent experiments.

3.3.4.6. Intracellular survival of other *A. butzleri* strains in *Acanthamoeba*

Both *A. butzleri* Arco-L and RM-4018 also manifested almost similar behaviour as that of ED-1 in *Acanthamoeba* (Figure 3.34). Both the strains had good internalization which was 0.7% and 0.6% for Arco-L and RM-4018 respectively but lesser than that of ED-1 (1.1%). Arco-L also showed proliferation by 2h in infection, however, it was not prominent for RM-4018. Moreover, RM-4018 had a big drop in intracellular bacterial number by only 4h as compared to ED-1 and Arco-L. There was no further proliferation beyond 2h period but survival at gradually declining low level.

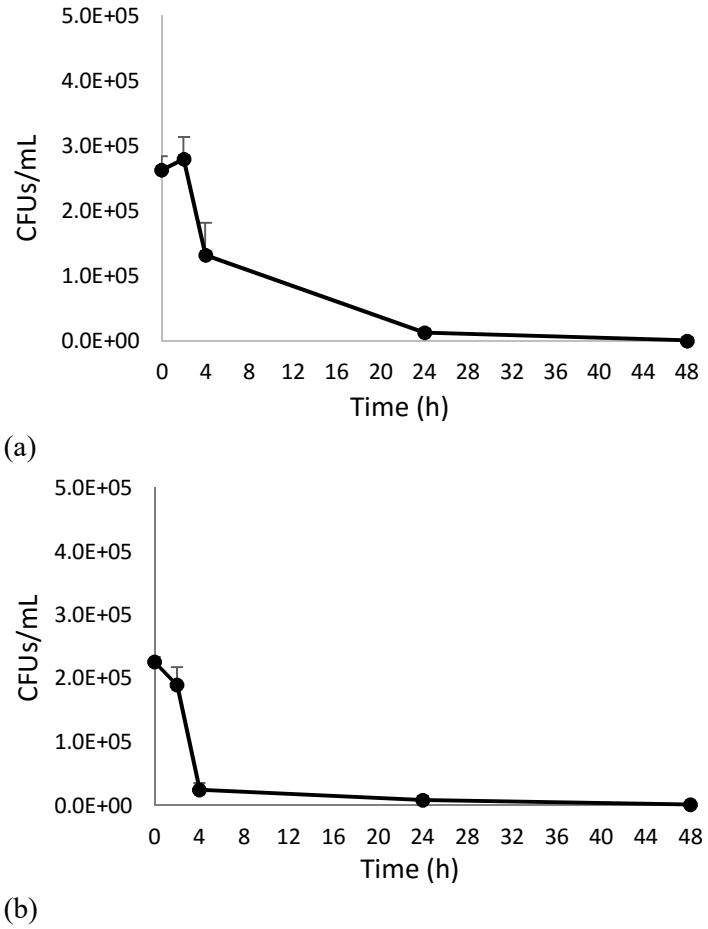


Figure 3.34: Intracellular survival pattern of *A. butzleri* Arco-L (a) and RM-4018 (b) in *A. castellanii* Neff over a period of 48h. Like the ED-1 strain of *A. butzleri*, the bacteria show some proliferation or better survival at 2h of infection followed by survival at lower levels. There are slight variations in the internalization abilities of the three strains by *Acanthamoeba*. The data represents mean±SE of three independent experiments.

It is interesting to note that the *A. butzleri* strain RM-4018 had the least internalization and also had the lowest survival capability in *Acanthamoeba* unlike the ED-1 strain. Therefore, the intracellular survival might be directly related to the internalization rate which means the more the bacteria are internalized (show survival at time zero), the better they have the survival in the cellular environment.

The intracellular survival patterns of *A. butzleri* strains indicate slight variations with the ED-1 strain which is also associated with the clinical cases being more aggressive for *Acanthamoeba* than others. *A. butzleri* ED-1 seems to be capable of defying the hostile killing mechanisms of the intracellular environment of *Acanthamoeba* and proliferate and survive. These findings indicate that *A. butzleri* can exploit

Acanthamoeba as environmental reservoirs. Survival beyond 48h was studied in long-term intracellular survival experiments (section 3.3.6.).

3.3.5. Pathogenic potential of *A. butzleri* towards *Acanthamoeba* (plaque assay)

A plaque assay was carried out using *A. castellanii* Neff and *A. butzleri* ED-1 to study the virulence potential of *A. butzleri* towards *Acanthamoeba* by measuring their spreading efficiency in cell monolayer. However, the plaques obtained were not only few but also very small (Figure 3.35). This indicates limited virulence capacity of *A. butzleri* for *Acanthamoeba* in terms of cell-to-cell spread.

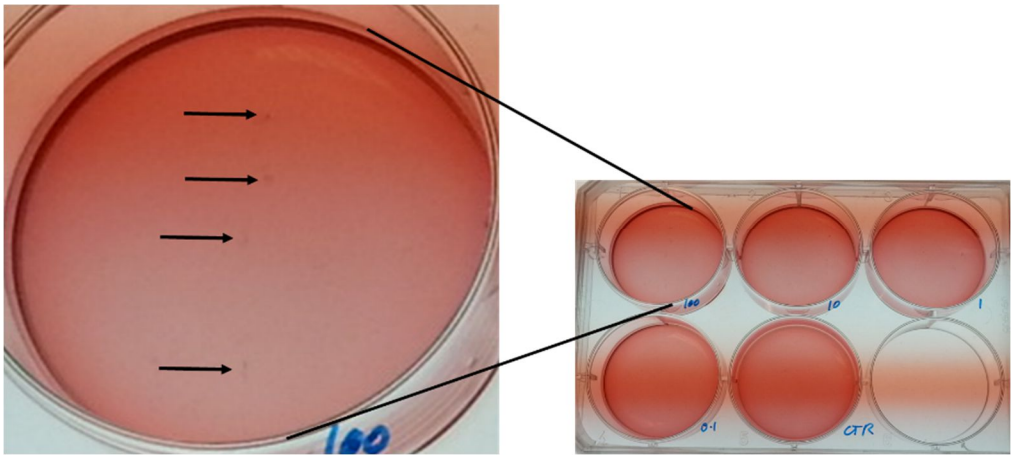


Figure 3.35: Formation of small plaques as a result of infection of *A. castellanii* Neff with *A. butzleri* ED-1 in a plaque assay. The plaques are very small and few. The cells were infected at various MOIs but the plaques were only seen in the well with highest MOI (100:1).

3.3.6. Impact of intra-amoebal survival of *A. butzleri* on their pathogenicity (re-infection assays)

The re-infection experiments were performed to assess the effect of repeated passages of *A. butzleri* through *Acanthamoeba* on the pathogenicity of these bacteria towards the latter. A total of 15 re-infection experiments were conducted and the effect on pathogenicity was assessed by undertaking a complete infection in *Acanthamoeba* (Neff). The effect was measured by comparing with control (infected with WT ED-1 bacteria) the increase in recovery of bacteria and the longevity of recovery.

3.3.6.1. Effect of recovered *A. butzleri* on their uptake and morphology

The data for internalization of bacteria at t=0 from 15 individual (Figure 3.36) as well as average re-infections (Figure 3.37-a) showed better recovery of bacteria in almost all the re-infection experiments. Furthermore, there was a clear difference in the morphological features of *A. butzleri* ED-1 after passage through the Neff cells (Figure 3.37-b,c). The recovered bacteria were different from the WT bacteria in two aspects; being shorter in length and more motile. Further investigation using electron microscopy may present more differences. These findings are significant and directly highlight the role of *Acanthamoeba* in inducing the pathogenic potential as a result of intracellular survival.

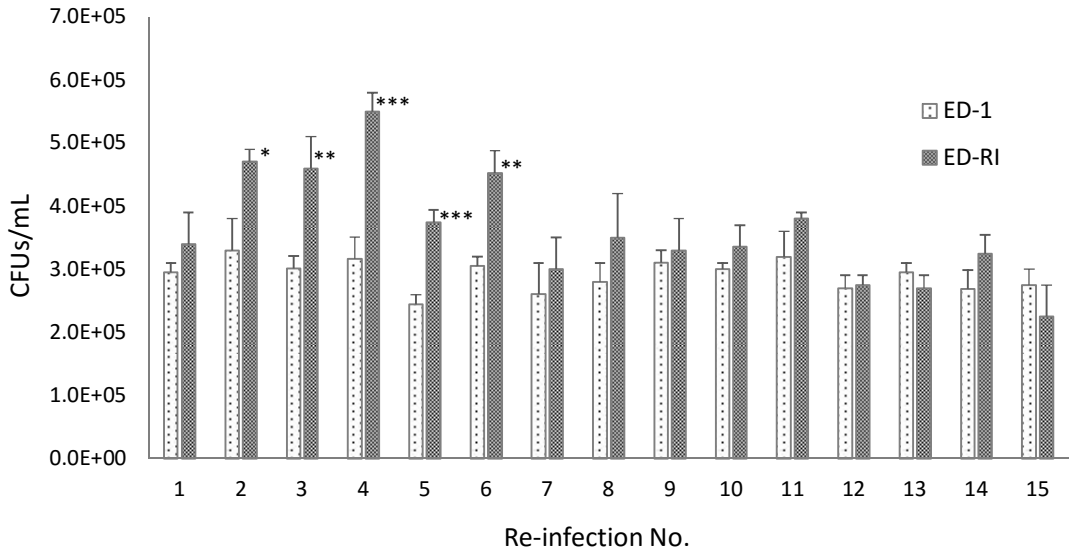


Figure 3.36: Effect of re-infections on internalization of *A. butzleri* ED-1 in *Acanthamoeba* Neff at t=0 (MOI=100:1) on individual re-infections in comparison with the WT ED-1 strain. The ED-RI represents the ED-1 bacteria recovered from the amoeba cells following an infection assay and these bacteria were then used for the next infection. The data, which represents mean±SE of the three re-infection experiments for each, was processed using Student's t-test (GraphPad). Asterisks (*) indicate significant difference at 95% confidence level compared to the paired WT ED-1 bacteria (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

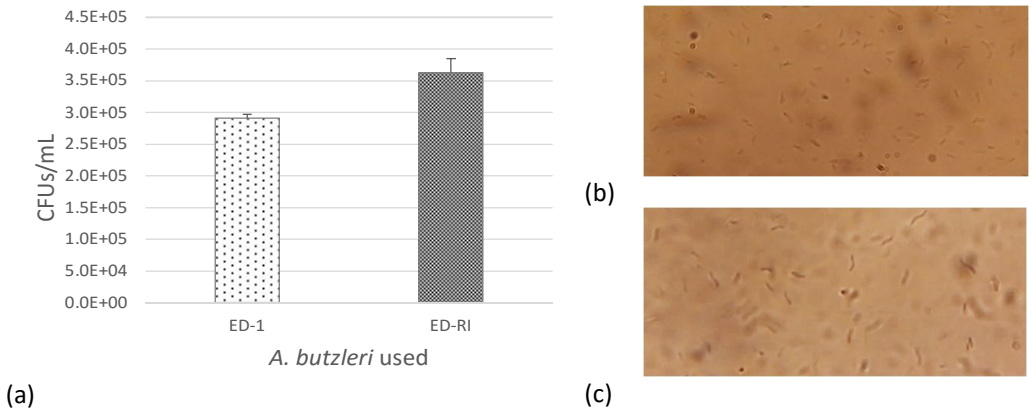
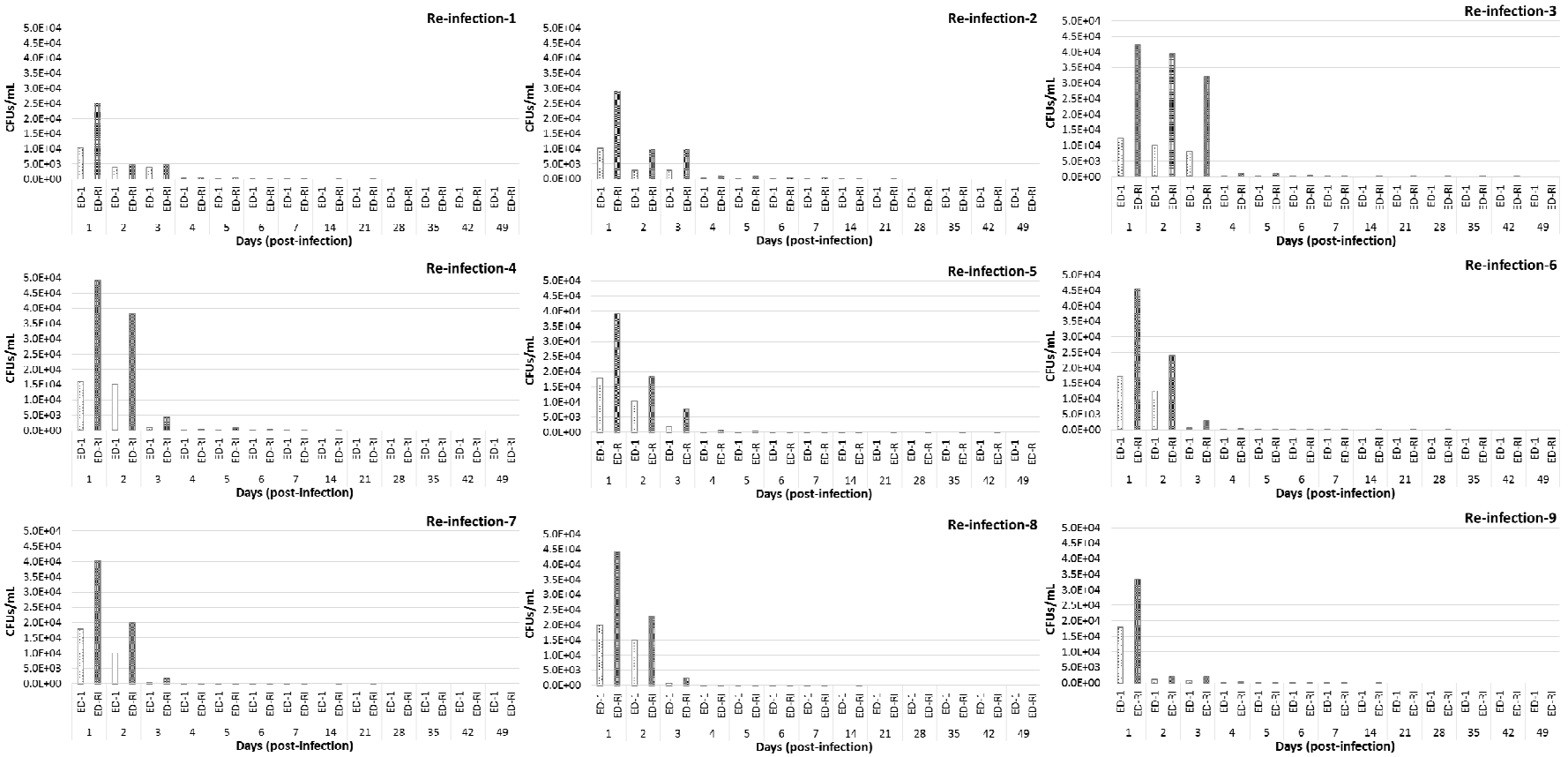


Figure 3.37: The impact of re-infections in *Acanthamoeba* on the virulence and morphology of *A. butzleri*. (a) Effect on internalization of A. butzleri ED-1 in *A. castellanii* Neff at t=0 using an MOI of 100:1. The data represents mean±SE of the 15 re-infection experiments. Using the Student's t-test (GraphPad) a significant effect on internalization was observed (p=0.005). (b) Recovered bacteria are smaller in length and more motile. (c) Normal WT ED-1 bacteria. The bacteria were cultured in VD media under microaerophilic conditions at 30°C with constant shaking and were observed live on glass slide with coverslip under microscope at 1000×.

3.3.6.2. Infection of *Acanthamoeba* with recovered bacteria

A detailed study was made using infection assays undertaken with the bacteria recovered from each of the re-infection experiments. A much better effect of recovered bacteria on enhanced internalization at time zero of infection was observed, compared to the control bacteria. The effect peaked during the initial six re-infections and it then started to decline in the later re-infections. Moreover, these bacteria showed much longer intracellular survival (upto 42 days post-infection) as shown in Figure 3.38.


Re-infection-1
Re-infection-2
Re-infection-3
Re-infection-4
Re-infection-5
Re-infection-6
Re-infection-7
Re-infection-8
Re-infection-9
CFUs/mL
Days (post-infection)

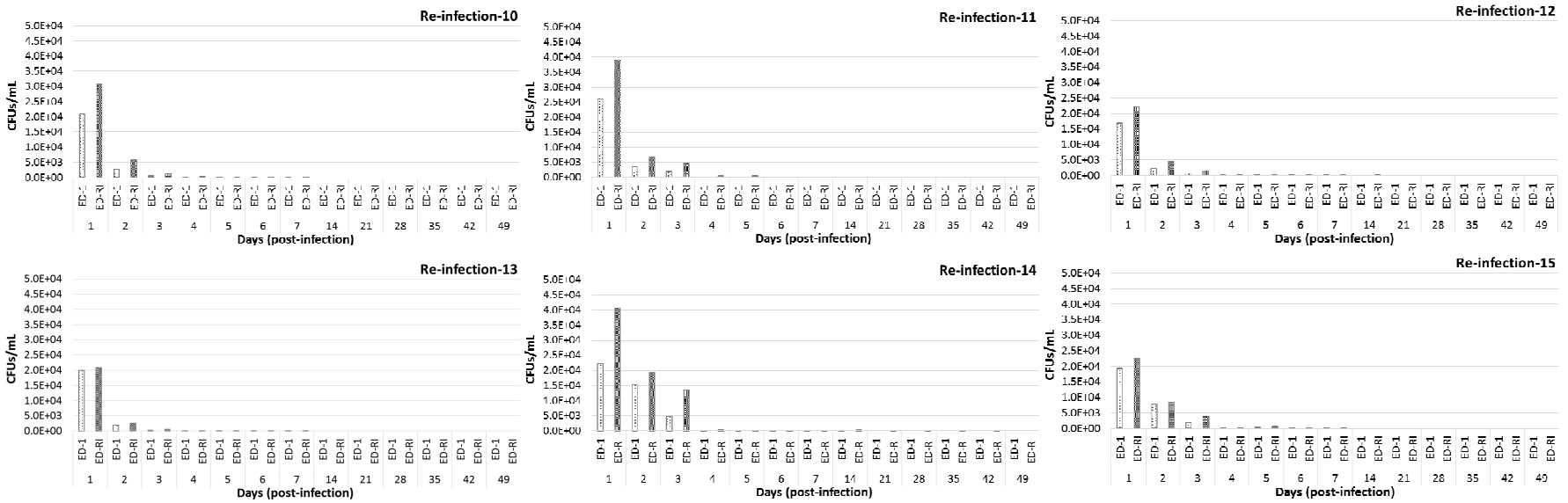


Figure 3.38: Detailed infection assays using *A. butzleri* ED-1 bacteria recovered from re-infection experiments (ED-RI) in parallel with normal ED-1 bacteria to see the effect of intracellular survival of *A. butzleri* in *Acanthamoeba* on its virulence. The bacteria recovered from one infection assay were used to start a new infection. A total of 15 re-infection experiments were conducted. Each re-infection experiment was performed upto 49 days using recovered bacteria (ED_RI) and normal bacteria (ED-1). Intracellular bacterial counts were made on day-0, 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 35, 42 and 49. ED-RI not only showed better survival at each time point but also demonstrated the longest intracellular survival (upto 42 days) compared to 14 days for ED-1. These results indicate that intracellular survival of *A. butzleri* in *Acanthamoeba* improves their infection capabilities.

Also a comparison was made between fold-change increase in the number of *A. butzleri* ED-1 bacteria recovered from re-infections at a given time point vs. the number of bacteria recovered from WT bacteria in control experiment in the same time point (Figure 3.39) which indicated a greater difference between the two in later infections which shows a better survival of recovered bacteria for longer times.

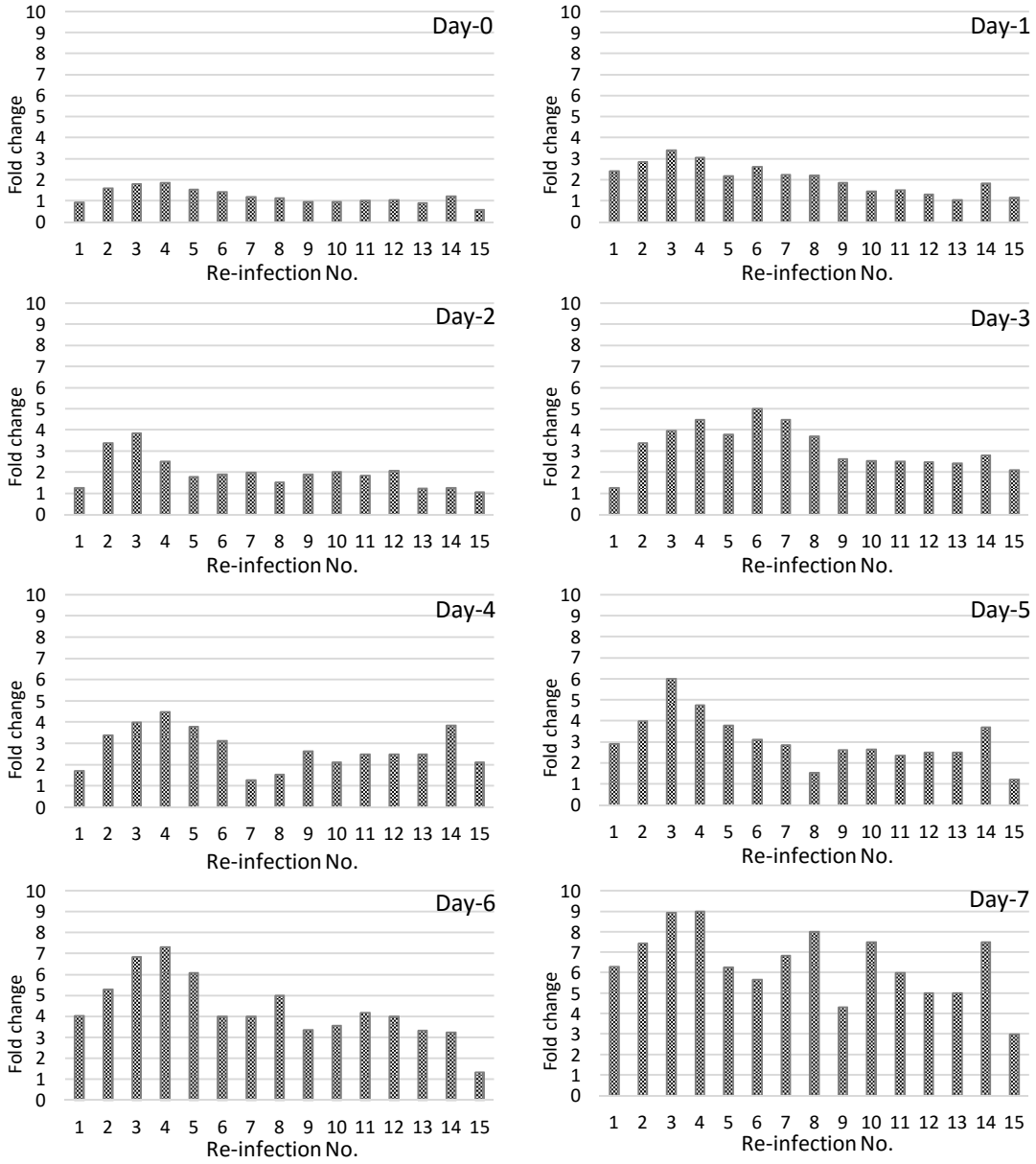


Figure 3.39: Fold-change in recovery of *A. butzleri* ED-1 for the recovered bacteria compared to the WT bacteria at each time point until day-7 for the 15 re-infections. This was achieved at any particular time point by dividing number of bacteria recovered from re-infection by the WT bacteria recovered at the same time point from control counterpart. As it can be seen in graphs from day-0 towards day-7, the difference increases. This is because of the declining number of control bacteria as compared to the bacteria from re-infection which show a longer and better survival of bacteria although at low number.

The results of re-infection experiments are very important as these indicate a potential role played by *Acanthamoeba* in induction of pathogenic traits as a result of survival

pressure within the hostile intracellular environment of these predators. Such interactions might be responsible for prominence of *A. butzleri* as emerging human pathogens.

3.3.7. Sensing the environmental change (increased nicotinic acid) and effect on virulence and survival of *A. butzleri* in *Acanthamoeba*

The exposure of *A. butzleri* to NA resulted in morphological changes in these bacteria. The NA-treated *A. butzleri* under the microscope appeared to be smaller and more motile than the non-treated bacteria. On using these bacteria for infecting *Acanthamoeba* cells, it was found that there were very obvious effects of NA treatment on the virulence of *A. butzleri* towards *Acanthamoeba*. Firstly, the infected cells with treated bacteria had significantly higher percentage of infected cells ($p<0.05$). Moreover, the number of *Acanthamoeba* cells lysed by t=0 of infection was also higher than that for the non-treated bacteria (Figure 3.40).

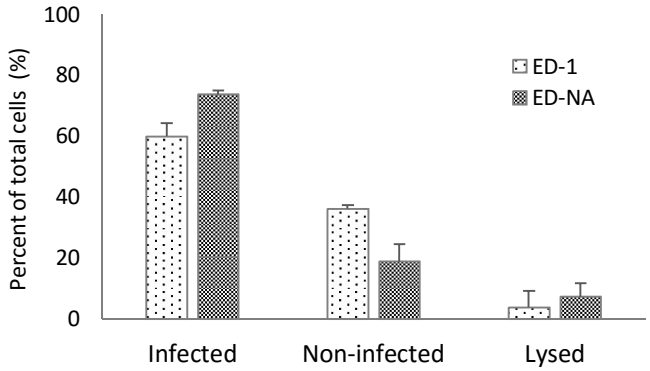


Figure 3.40: Effect of nicotinic acid (5 mM) treated *A. butzleri* ED-1 on percent infection and lysis of *A. castellanii* Neff. Parallel infections were also made with non-treated ED-1. Treated amoeba cells had significantly higher population of infected cells compared to the non-treated cells ($p<0.05$ at 95% confidence level, calculated by Student's t-test using GraphPad). The data represents mean±SE of two independent experiments.

Complete infection assays were performed using the treated and non-treated *A. butzleri* bacteria and the effect on internalization and the survival of bacteria in the *Acanthamoeba* was also studied over a period of 48h. Interestingly, there was a significantly higher internalization at t=0 of the infection for the treated bacteria as compared to the non-treated bacteria. The bacterial attachment caps on *Acanthamoeba* cells were wider. However, at the subsequent time points there was no significant difference in the number of bacteria recovered from the treated and non-treated groups. Also there was no effect of NA treatment on the longer survival of bacteria inside *Acanthamoeba* (Figure 3.41).

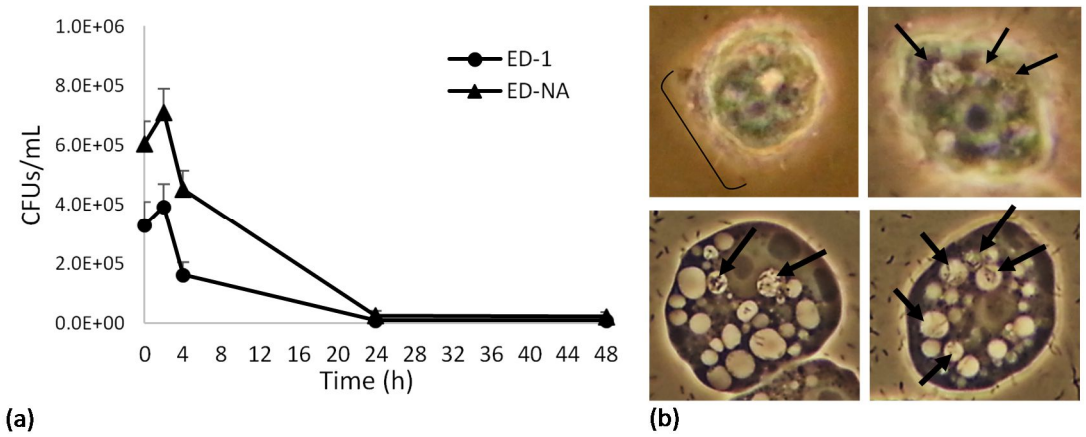


Figure 3.41: Effect of nicotinic acid (NA) treatment on intracellular survival of *A. butzleri* ED-1 in *Acanthamoeba*. Infection dynamics of *A. castellanii* Neff with *A. butzleri* ED-1 that were treated with NA (5 mM) in parallel with non-treated ED-1 (a). The data represents mean±SE of six independent experiments. Cells infected with treated bacteria form bigger cap on the surface of the cell (bracket) and are heavily infected which are indicated by arrows (b).

The infection of *Acanthamoeba* cells with NA-treated bacteria had a gross morphological effect on cells shapes which appeared to have uncontrolled actin polymerization and pseudopod formation giving rise to bleb-like structures (Figure 3.42). These results indicate an active role of the bacterial two-component system in sensing the environmental changes (in the form of increased NA concentration) and modulating their phenotypic and pathogenic profile in the new environment. Taken together, the enhanced virulence and the role of amoebae in increasing the pathogenic potential of bacteria may have consequences for this emerging human pathogen in expanding its pathogenic capabilities towards humans.

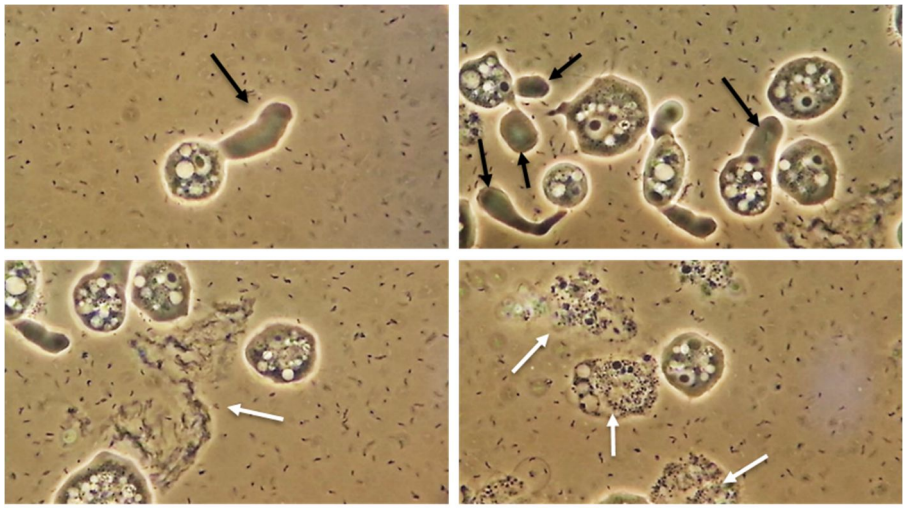


Figure 3.42: The effect of NA treatment of *A. butzleri* ED-1 on *A. castellanii* Neff when infected with these bacteria. The infection caused obvious morphological changes in the *Acanthamoeba* cells. The cells appeared to have uncontrolled actin polymerization changes in the form of long and irregular cellular extensions or blebs (black arrows). The cells appeared heavily infected than the non-treated control cells and the dead cells (white arrows) can be seen clearly.

3.3.8. Effect of living together-the co-cultures of *A. butzleri* and *Acanthamoeba*

Co-cultures of *Acanthamoeba* and *A. butzleri* were established to investigate the mutual impact on both the organisms. The co-cultures were studied under different setups and conditions. The simple qualitative form of co-culture was studied on agar plates with overlaid live *A. butzleri* onto which spots of *Acanthamoeba* with variable numbers were placed. The qualitative co-cultures were performed in different liquid media with continuous monitoring of both the organisms.

3.3.8.1. Co-culture on agar overlaid with live *A. butzleri*

In this qualitative setup, *Acanthamoeba* and *A. butzleri* were co-cultured on a non-nutrient agar plate with overlaid live washed *A. butzleri* ED-1 in saline with low levels of VD media. *Acanthamoeba* were spotted on agar with ascending concentration levels (2×10^2 to 1×10^5). The time-lapse photography results of the lowest and highest concentration are presented in Figure 3.43 and 3.44. Bacteria can be seen being engulfed and wiped out, however, the process of engulfing bacteria was much slower with lower concentration of *Acanthamoeba* than the higher.

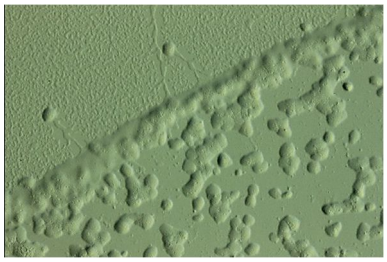


Figure 3.43: *A. castellanii* Neff at low concentration (2×10^2) and live *A. butzleri* ED-1 in co-culture on agar plate. The speed of bacterial engulfment and *Acanthamoeba* growth was much slower than when *Acanthamoeba* were in higher concentration.

The response of the *Acanthamoeba* Neff at high concentration of cells was quicker than lower concentration and the *Acanthamoeba* and the amoebal growth front could be seen moving forward leaving behind the clear zone. Slower growth of amoeba cells at lower concentration while faster growth at high concentration of amoebal cells indicates resistance of *A. butzleri* in the former situation which is reduced when the amoebal number is much higher. These quantitative experiments, therefore, indicate certain degree of resistance to killing by *Acanthamoeba* cells.

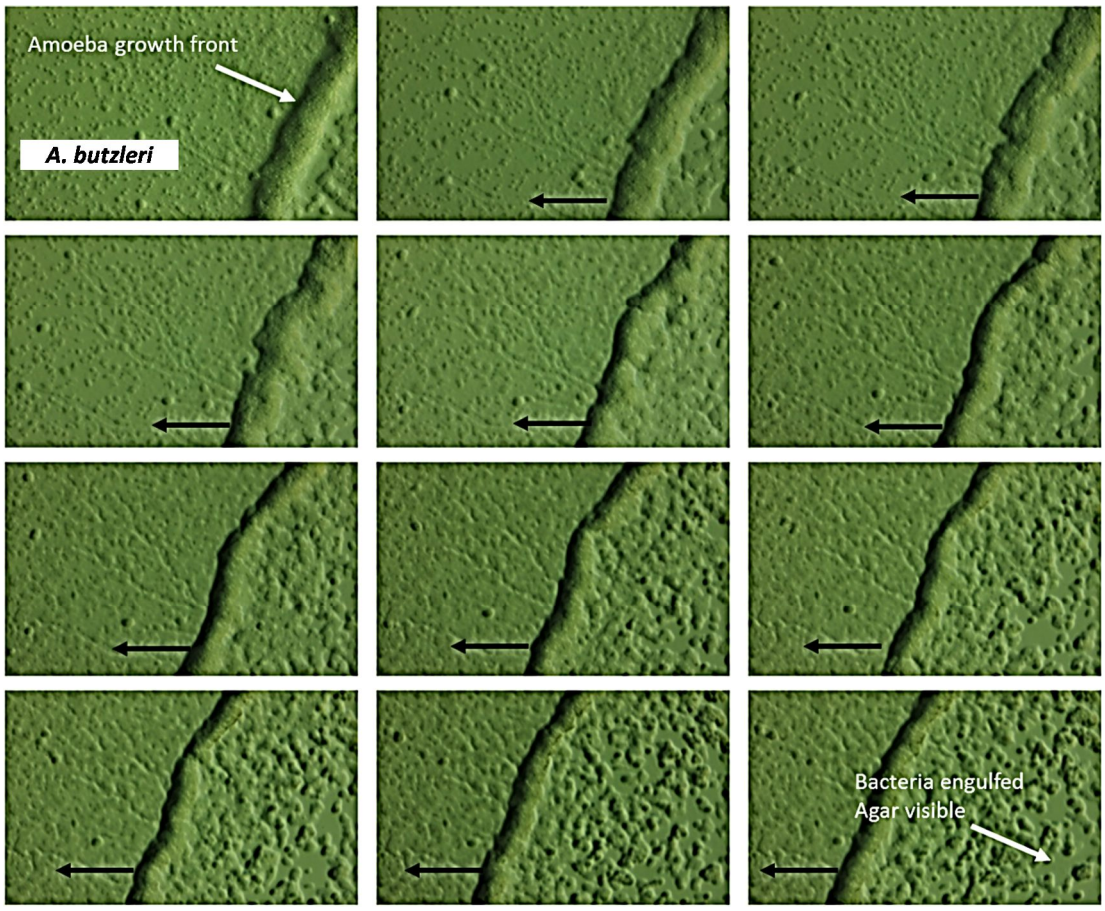


Figure 3.44: Time-lapse photographic sequences of co-culture on an agar plate between *A. castellanii* Neff (1×10^5) and live *A. butzleri* ED-1 overlaid on agar plate. *Acanthamoeba* can be seen engulfing the bacteria and growing out leaving clear agar behind. The clearing up of bacteria was quicker as compared to that at lower concentration of *Acanthamoeba* probably because of decrease in number of bacteria available per cell which results in lesser bacteria internalized and hence fewer chances of survival and amoebal cell killing.

Although these experiments didn't indicate the exact fate of *Acanthamoeba*, one thing was clear that *A. butzleri* didn't cause massive cell lysis of *Acanthamoeba* which was comparable with the finding of low levels of cellular lysis caused by infection of these bacteria in infection assays (Figure 3.28). Quicker engulfment of *A. butzleri* at higher concentration of *Acanthamoeba* cells also indicates a dose-dependent fate of these bacteria. This was comparable with the finding that intracellular survival of *A. butzleri* is related with ability of these bacteria to successfully become internalized into the cells at a good number. At much higher number of *Acanthamoeba*, the number of bacteria available per amoeba cell decreases and probably for that reason enough bacteria are not present in a cell to sustain survival for longer or to kill the cell (section 3.3.4.4.). The effect of co-culture of *Acanthamoeba* and *A. butzleri* was studied quantitatively under different conditions as laid out in Figure 3.45 and the following details.

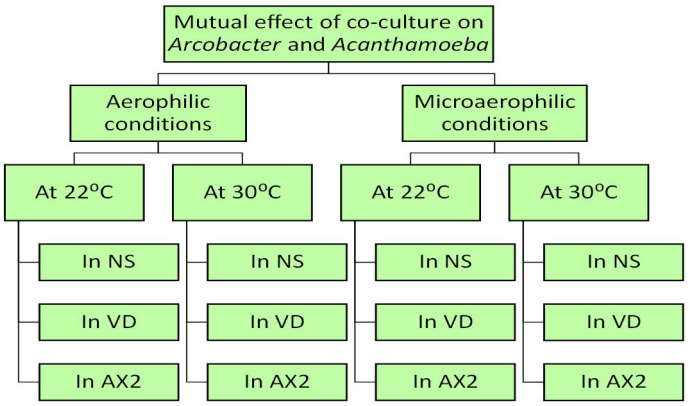


Figure 3.45: Overview of the layout of co-culture studies of *Acanthamoeba* Neff and *A. butzleri* ED-1 ender different liquid media at either 22ºC or 30ºC and either under aerophilic or microaerophilic conditions.

3.3.8.2. Effect of *A. butzleri* on *Acanthamoeba* in co-culture

The effect of co-culture on *Acanthamoeba* was measured separately when co-culture was undertaken in NS, VD and AX2+ under either aerophilic or microaerophilic conditions and either at 22°C or 30°C. The results are presented separately for NS, VD and AX2+. The effect of co-culture on *Acanthamoeba* was assessed on the basis of cellular growth and the degree of encystation.

3.3.8.2.1. Effect of *A. butzleri* on *Acanthamoeba* in NS

3.3.8.2.1.1. Under aerophilic conditions and 22ºC vs 30ºC

In NS, at both 22ºC and 30ºC, no proliferation of *Acanthamoeba* was observed in the uninfected control group, rather there was a gradual drop in the number of trophozoites over the period of 96h (Figure 3.46). This was obviously due to lack of food available for the cells to grow. The total number of cells (trophozoites+cysts) almost remained the same with a minor decline by 96h. However, in the infected group there was even a further drop in the trophozoites and cysts. Microscopically, at 30ºC comparatively more rounded cells and cysts were seen than the cells at 22ºC which also appeared bigger in size (Figure 3.47). Temperature itself, therefore, appeared to have effect on rounding off the cells as the number of trophozoites dropped sharply by 24h in case of 30ºC cultures. This showed that increased temperature has negative effects on growth of *Acanthamoeba* when grown in NS. Furthermore, at both the temperatures, there was a comparative decrease in number of trophozoites and total number of cells in the infected group (a decline from 1.0×10^6 to 8.2×10^5 in case of 22ºC control and to 7.4×10^5 for 22ºC infected). Similarly in case of 30ºC cultures there was a decline from 1.0×10^6 to 7.6×10^5 and 6.6×10^5 for control and infected groups respectively. This indicates a pathogenic role of these bacteria in co-culture in NS.

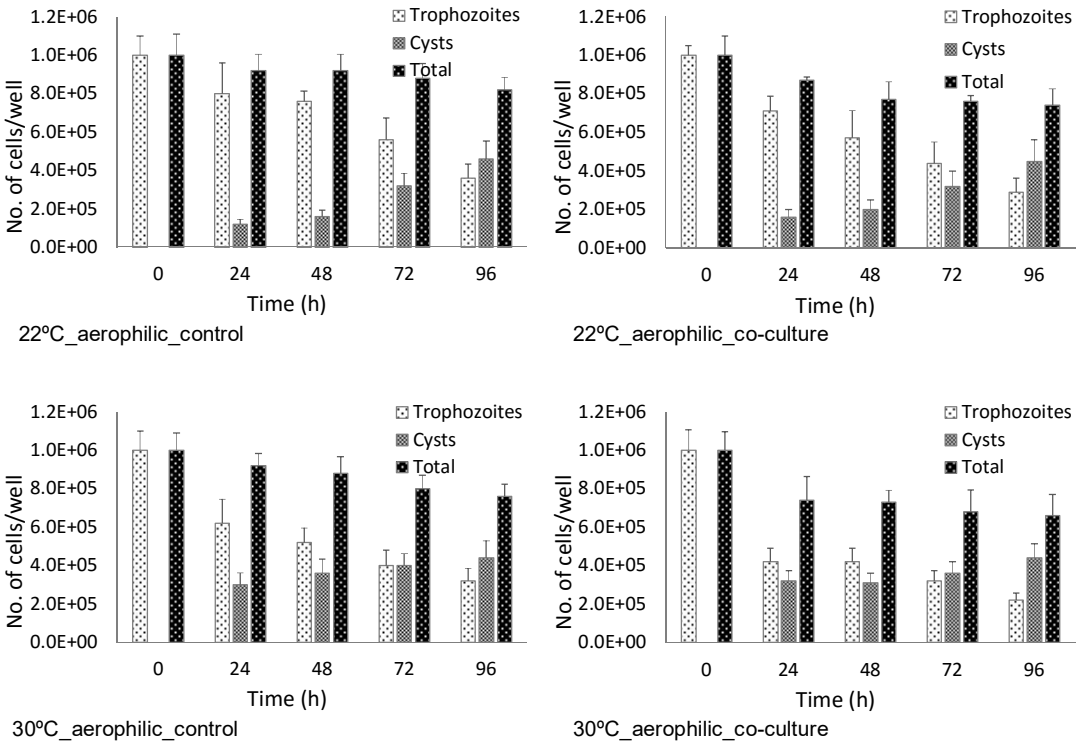


Figure 3.46: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **NS** under **aerophilic conditions** on *Acanthamoeba* over a period of 96h. The co-culture was established under different growth conditions as mentioned with each graph. *Acanthamoeba* did not show any growth even in solo culture at both the temperatures because of the nutrient deficient NS. This also indicates that they did not get any support from *A. butzleri* in the form of some released factors that might support growth of *Acanthamoeba*. Rather the *Acanthamoeba* in co-cultures had slightly lower total cell number. The data represents mean±SE of two independent experiments.

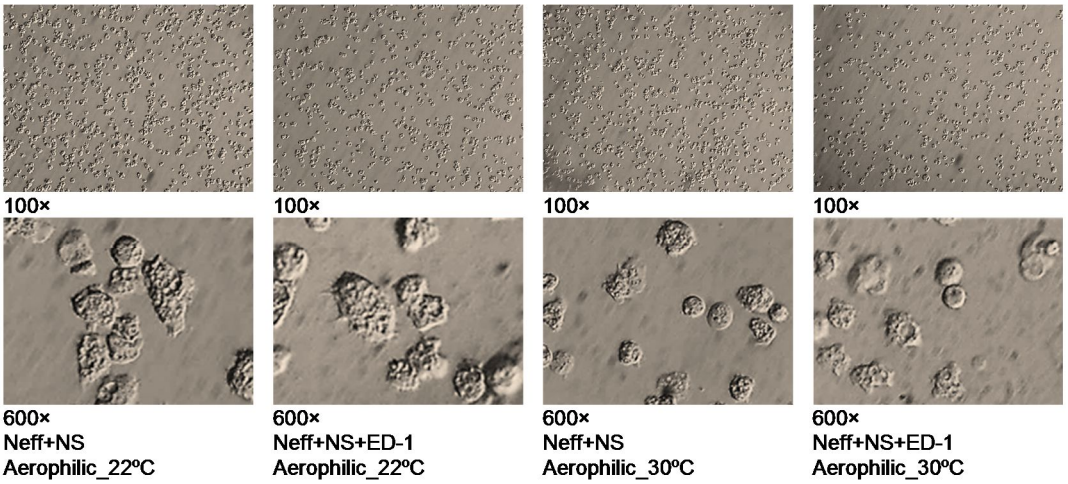


Figure 3.47: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **NS** under **aerophilic conditions** on *Acanthamoeba* by 24h. The co-culture was established under different growth conditions as mentioned with each row of pictures. *Acanthamoeba* did not show any growth even in solo culture at both the temperatures because of the nutrient deficient NS. Cells at 30ºC had even poorer condition.

3.3.8.3.1.2. Under microaerophilic conditions and 22ºC vs 30ºC

Overall, there was similar trend in case of microaerophilic cultures, as at each time point the total number of cells for the infected group was lesser compared to the uninfected control group, indicating the pathogenic potential of *A. butzleri* towards *Acanthamoeba* due to lesser integrity of these cells (Figure 3.48). The total number of cells declined from initial number of 1.0×10^6 to 7.8×10^5 and 6.2 for 22ºC control and infected group, respectively. Similarly there was a drop of cells from initial number of 1.0×10^6 to 7.7×10^5 and 6.3×10^5 for 30ºC control and infected group, respectively. The microaerophilic conditions also seemed to have an effect on the *Acanthamoeba* cells as the cells started to round off by 24h (Figure 3.49).

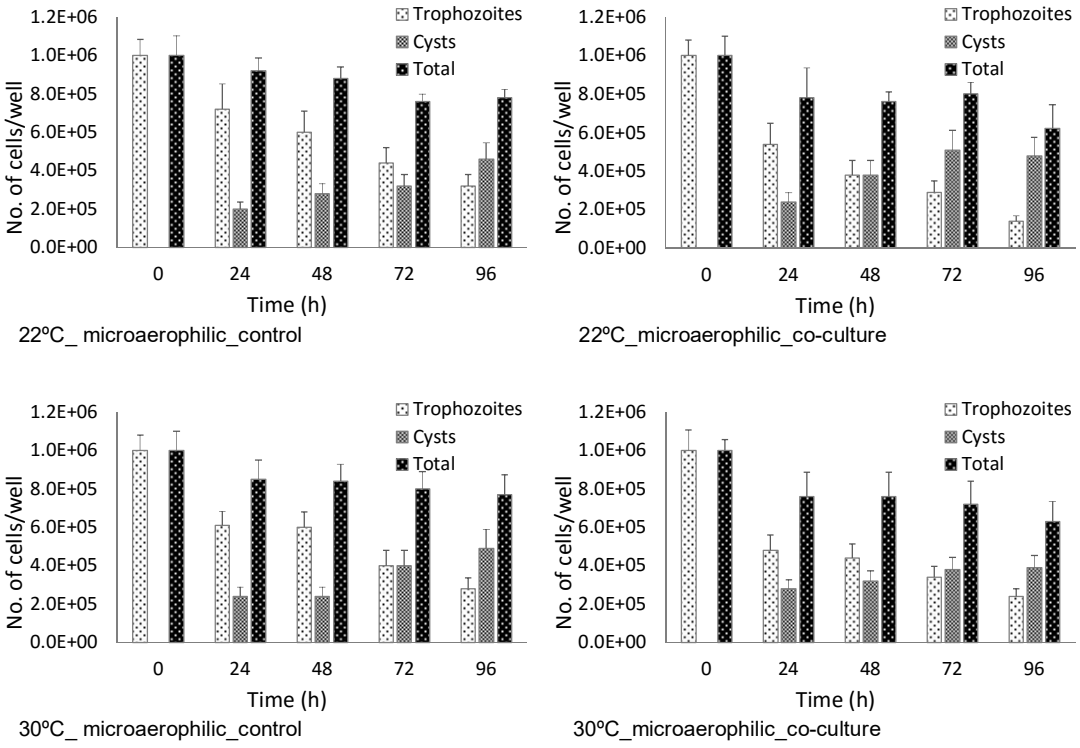


Figure 3.48: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **NS** under **microaerophilic conditions** on *Acanthamoeba* over a period of 96h. The co-culture was established under different growth conditions as mentioned with each graph. *Acanthamoeba* did not show any growth even in solo culture at both the temperatures because of the nutrient deficient NS. This also indicates that they did not get any support from *A. butzleri* in the form of some released factors that might support growth of *Acanthamoeba*. Rather the *Acanthamoeba* in co-cultures had slightly lower total cell number. The data represents mean ±SE of two independent experiments.

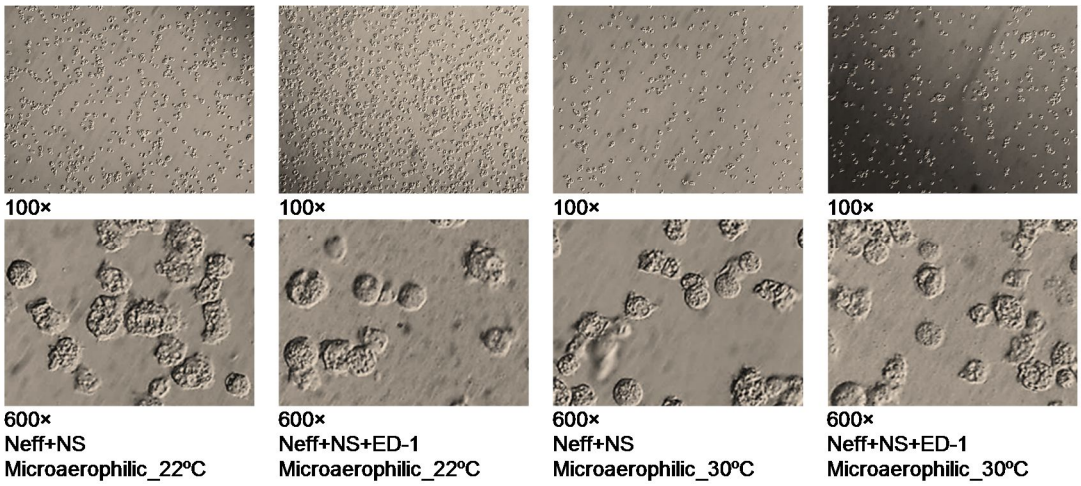


Figure 3.49: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **NS** under **microaerophilic conditions** on *Acanthamoeba* by 24h. The co-culture was established under different growth conditions as mentioned with each row of pictures. *Acanthamoeba* did not show any growth even in solo culture at both the temperatures because of the nutrient deficient NS. Cells at 30ºC had even poorer condition.

Overall, the *Acanthamoeba* cells do not seem to be benefited by the co-culture in saline, rather their total number goes slightly down as a result of co-culture which appears to be due to the pathogenic nature of *A. butzleri* towards *Acanthamoeba*, although the affected number of *Acanthamoeba* cells a very small.

3.3.8.3.2. Effect of *A. butzleri* on *Acanthamoeba* in VD

3.3.8.3.2.1. Under aerophilic conditions and 22ºC vs 30ºC

The *Acanthamoeba* non-infected control cells showed a good growth in VD media, although it was not as good as in AX2+. However, *Acanthamoeba* in the infected groups at both 22ºC and 30ºC failed to grow and the total number of cells gradually faded over the period of 96h (Figure 3.50). This indicates pathogenic/inhibitory effect of *A. butzleri* towards *Acanthamoeba* which is not affected by temperatures used. The *Acanthamoeba* cells at both the temperatures looked healthier as compared to the infected cells which turned round and appeared to be stressed (Figure 3.51).

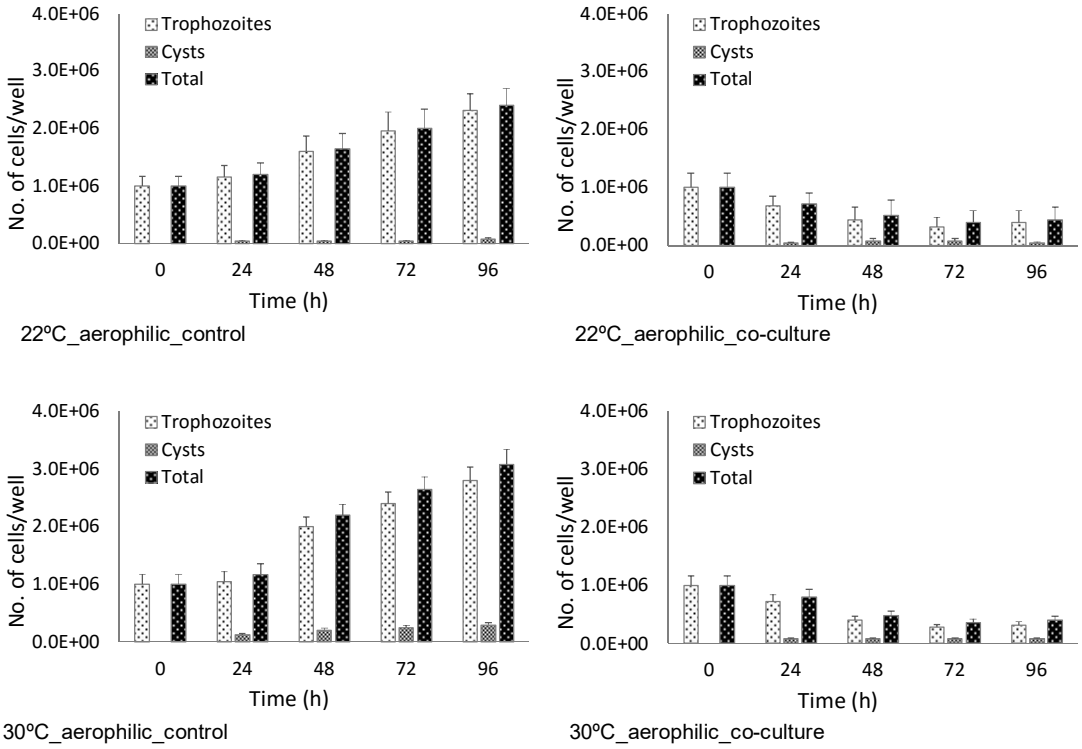


Figure 3.50: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **VD** under **aerophilic conditions** on *Acanthamoeba* over a period of 96h. The co-culture was established under different growth conditions as mentioned with each graph. Unlike co-culture in NS, in VD *Acanthamoeba* did show growth in solo culture at both the temperatures. However, there was no growth in case of co-cultures indicating an inhibitory role of *A. butzleri* towards *Acanthamoeba* in co-cultures. The data represents mean±SE of two independent experiments.

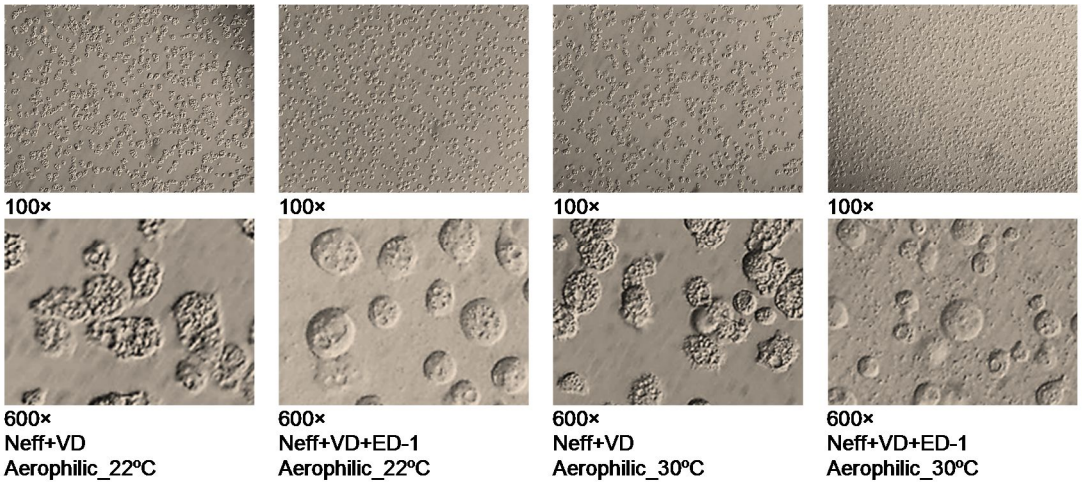


Figure 3.51: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **VD** under **aerophilic conditions** on *Acanthamoeba* by 24h. The co-culture was established under different growth conditions as mentioned with each row of pictures. *Acanthamoeba* did not have a good growth in solo culture at both the temperatures because VD is not a good media for *Acanthamoeba*. Furthermore, *A. butzleri* in the co-culture had even poorer growth because of the negative impact of *A. butzleri* on the growth and integrity of *Acanthamoeba* cells as evident from these pictures.

3.3.8.3.2.1. Under microaerophilic conditions and 22°C vs 30°C

The un-infected *Acanthamoeba* cells in VD media under microaerophilic condition although showed some growth, the growth was affected further at 30ºC. Compared to the control group, the infected cells had even further drop in the number of cells (Figure 3.52). This observation further strengthens the finding that *A. butzleri* have inhibitory effect on the growth of *Acanthamoeba*. Microscopically, the cells in control groups appeared normal but with lot of vacuolation. Interestingly, the rounding off phenomenon of *Acanthamoeba* in the infected cells was lesser as compared to the aerophilic group (Figure 3.53).

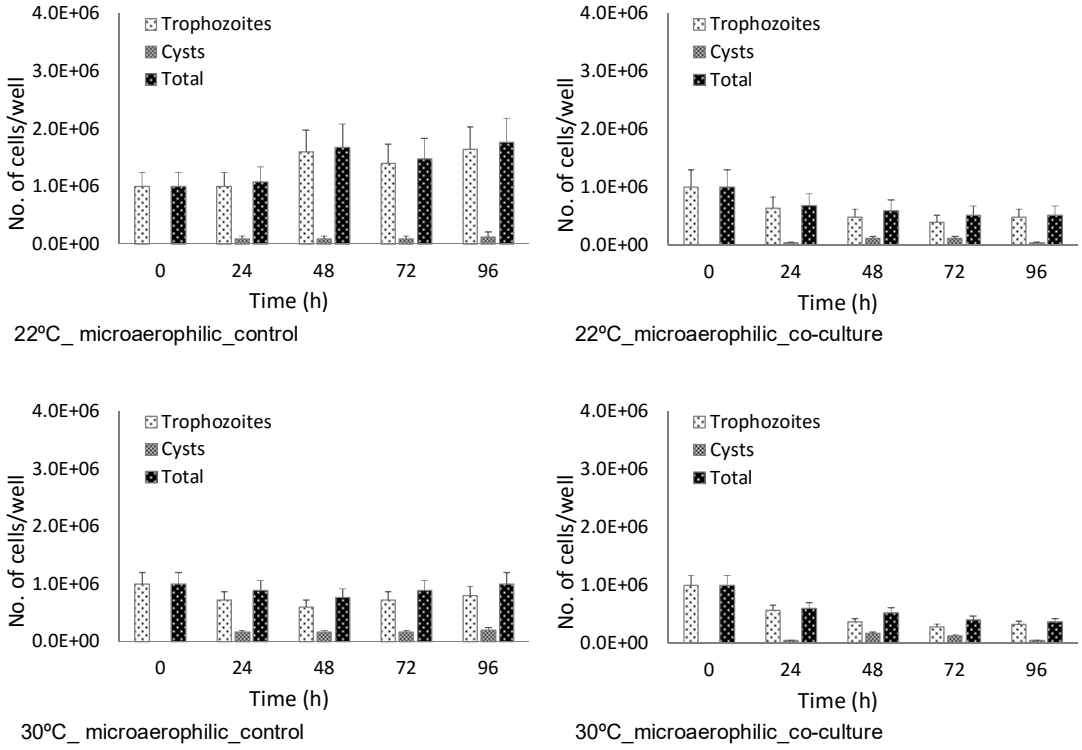


Figure 3.52: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **VD** under **microaerophilic conditions** on *Acanthamoeba* over a period of 96h. The co-culture was established under different growth conditions as mentioned with each graph. Unlike co-culture in NS, in VD *Acanthamoeba* did show growth in solo culture at both the temperatures although it was lesser than the one in VD under aerophilic conditions. There was no growth in case of co-cultures indicating an inhibitory role of *A. butzleri* towards *Acanthamoeba* in co-cultures. The data represents mean±SE of two independent experiments.

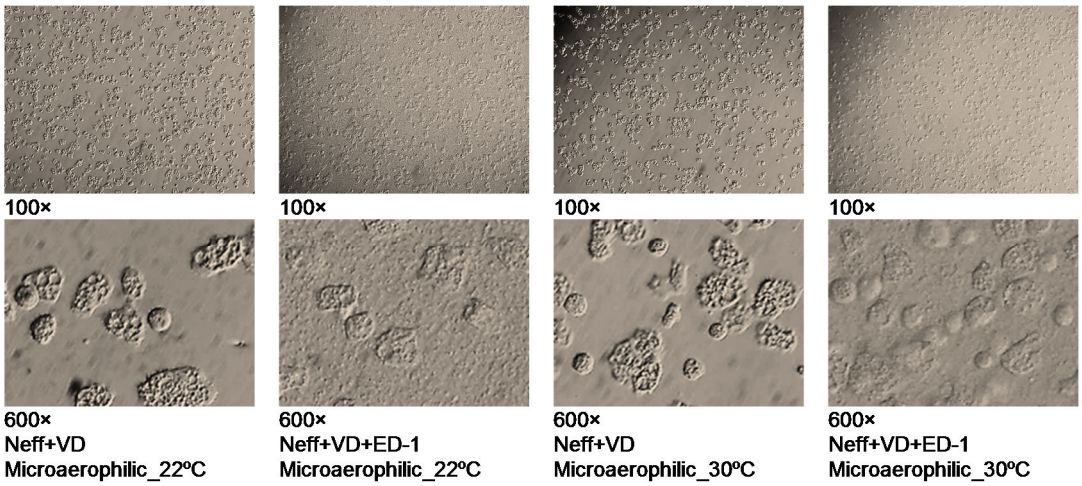


Figure 3.53: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **VD** under **microaerophilic conditions** on *Acanthamoeba* by 24h. The co-culture was established under different growth conditions as mentioned with each row of pictures. *Acanthamoeba* did not have a good growth in solo culture at both the temperatures because VD is not a good media for *Acanthamoeba*. Furthermore, *A. butzleri* in the co-culture had even poorer growth because of the negative impact of *A. butzleri* on the growth and integrity of *Acanthamoeba* cells as evident from these pictures.

3.3.8.3.3. Effect of *A. butzleri* on *Acanthamoeba* in AX2+

3.3.8.3.3.1. Under aerophilic conditions and 22ºC vs 30ºC

The uninfected cells at 22ºC showed a very good growth in AX2+ but this was affected significantly at 30ºC. Interestingly, the growth of *Acanthamoeba* in infected cultures was significantly retarded at both the temperatures (Figure 3.54). This observation further reinforces the finding that *A. butzleri* may have an inhibitory effect on the growth of *Acanthamoeba*. Another finding was that unlike the co-cultures in NS and VD, the *Acanthamoeba* cells in AX2+ by 24h did not round off extensively even in the presence of *A. butzleri* (Figure 3.55).

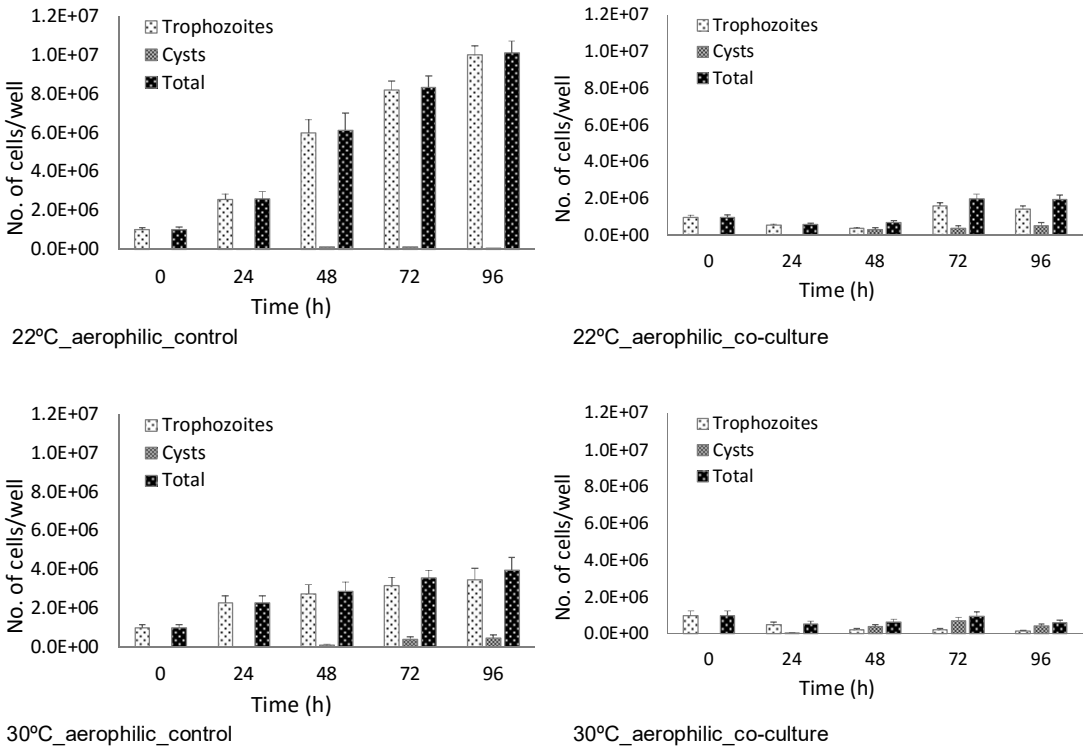


Figure 3.54: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **AX2+** under **aerophilic conditions** on *Acanthamoeba* over a period of 96h. The co-culture was established under different growth conditions as mentioned with each graph. Unlike co-culture in NS or VD, *Acanthamoeba* showed massive growth in solo culture at 22ºC whereas the growth was lesser in solo 30ºC as it is less favourable temperature for *Acanthamoeba*. There was significantly low growth in case of co-cultures indicating an inhibitory role of *A. butzleri* towards *Acanthamoeba* in co-cultures. The data represents mean±SE of two independent experiments.

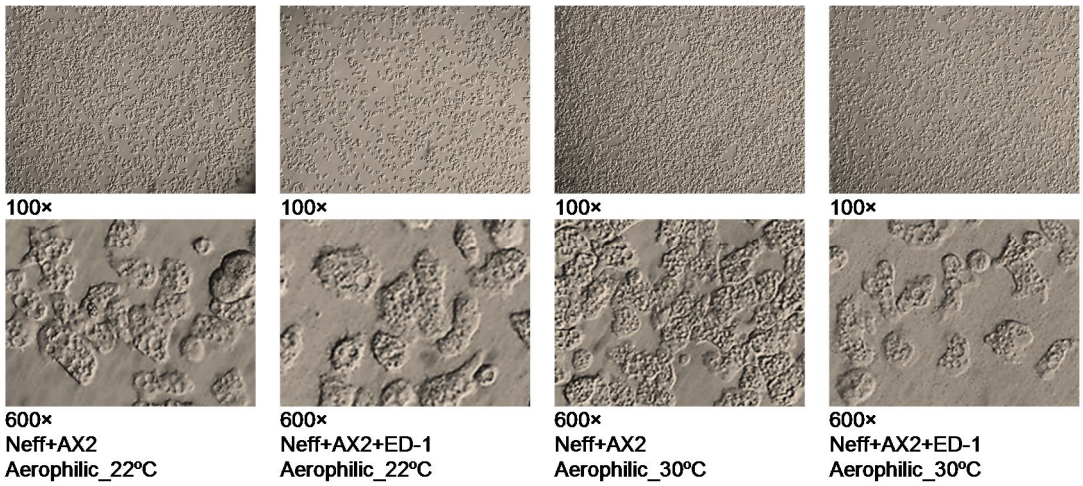


Figure 3.55: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **AX2+** under **aerophilic conditions** on *Acanthamoeba* by 24h. The co-culture was established under different growth conditions as mentioned with each row of pictures. *Acanthamoeba* had a very good growth in solo culture at both the temperatures as the aerophilic conditions and AX2+ are both favourable for *Acanthamoeba*. However, *A. butzleri* in the co-culture seemed to have a negative impact on the growth and integrity of *Acanthamoeba* cells as evident from these pictures.

3.3.8.3.3.2. Under microaerophilic conditions and 22°C vs 30°C

The uninfected *Acanthamoeba* cells showed a good growth even under microaerophilic conditions although the growth was lower than in aerophilic conditions. But importantly the similar inhibitory effect of *A. butzleri* was observed against *Acanthamoeba* which resulted in significantly lower growth of *Acanthamoeba* in co-cultures (Figure 3.56). The cellular rounding off phenomenon was observed under microaerophilic conditions in infected cells only by 24h (Figure 3.57). This might be due to additive stress of lower oxygen and inhibitory effect of *A. butzleri*.

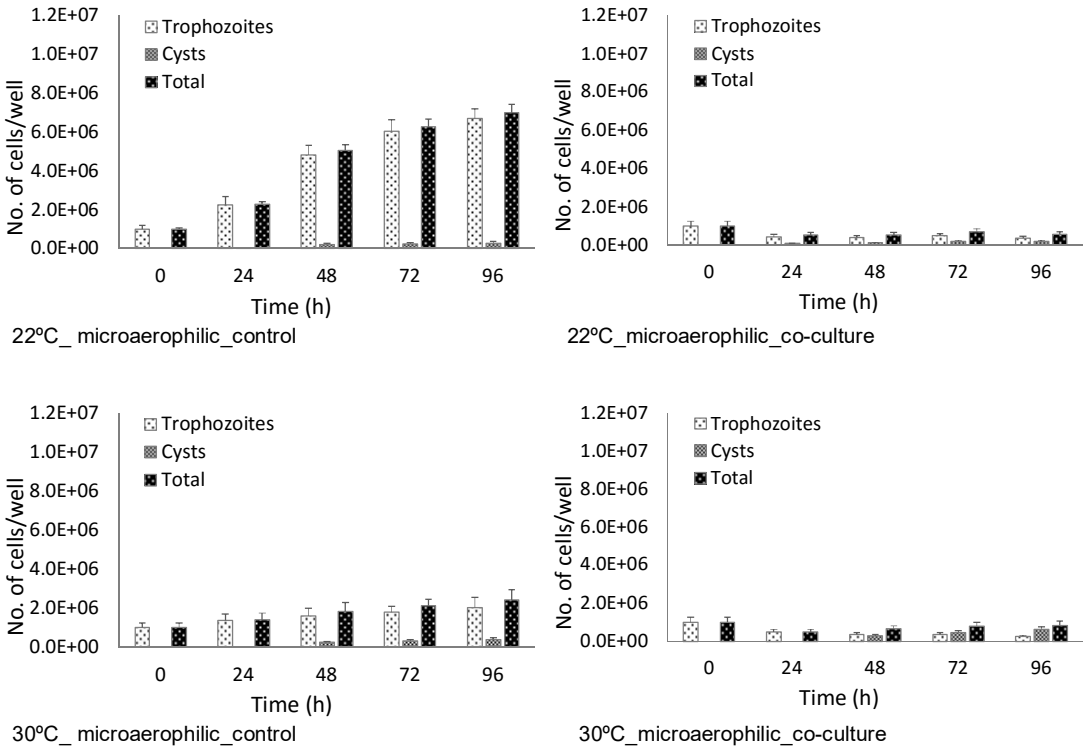


Figure 3.56: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **AX2+** under **microaerophilic conditions** on *Acanthamoeba* over a period of 96h. The co-culture was established under different growth conditions as mentioned with each graph. Unlike co-culture in NS or VD, *Acanthamoeba* showed massive growth in solo culture at 22ºC whereas the growth was lesser in solo 30ºC as it is less favourable temperature for *Acanthamoeba*. There was significantly low growth in case of co-cultures indicating an inhibitory role of *A. butzleri* towards *Acanthamoeba* in co-cultures. The data represents mean±SE of two independent experiments.

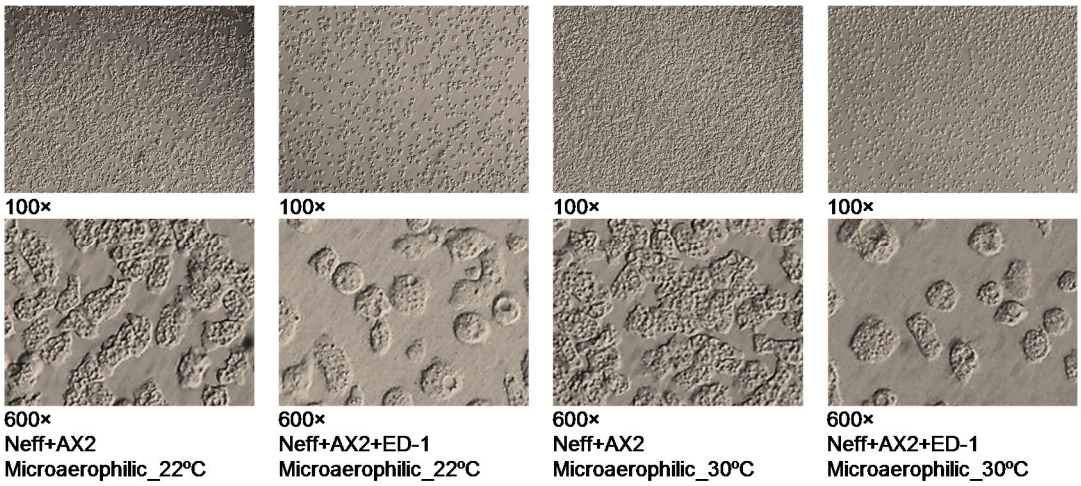


Figure 3.57: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **AX2+** under **microaerophilic conditions** on *Acanthamoeba* by 24h. The co-culture was established under different growth conditions as mentioned with each row of pictures. *Acanthamoeba* had a very good growth in solo culture at both the temperatures. However, *A. butzleri* in the co-culture seemed to have a negative impact on the growth and integrity of *Acanthamoeba* cells as evident from these pictures.

3.3.8.3. Effect of *Acanthamoeba* on *A. butzleri* in co-culture

The effect of *Acanthamoeba* on *A. butzleri* was also quantitatively measured under similar conditions of growth and culture as used for studying the effect of *A. butzleri* on *Acanthamoeba*. This was measured on the basis of number of *A. butzleri* in the culture. The results are presented below.

3.3.8.3.1. Effect of *Acanthamoeba* on *A. butzleri* in NS

A. butzleri in co-culture in NS did not seem to be benefited by *Acanthamoeba* as their number gradully declined over the 96h time period (Figure 3.58). This might be due to lack of nutrients causing stress on both the organisms. Furthermore, the inhibitory effect of *A. butzleri* towards *Acanthamoeba* probabaly further stressed the *Acanthamoeba* cells forcing them to encyst and being incapable of producing any factors that might be supportive for *A. butzleri* growth.

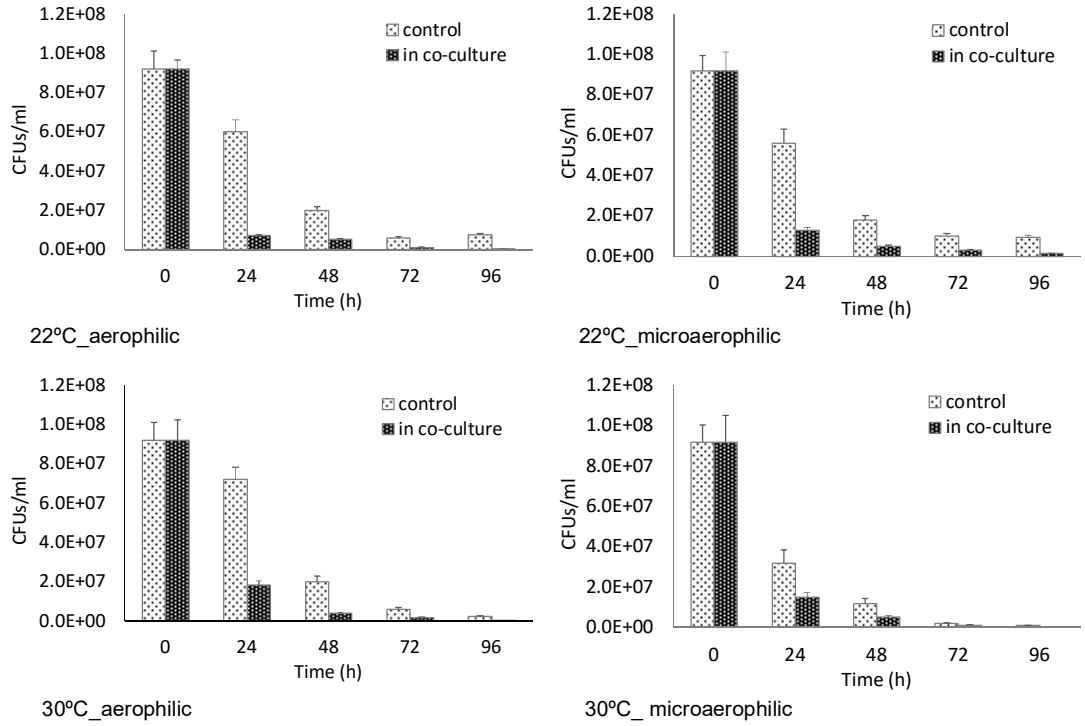


Figure 3.58: Effect of co-culture of *Acanthamoeba* Neff and *A. butzleri* ED-1 in **NS** on *A. butzleri* ED-1. The co-culture was established under different growth conditions as mentioned under each graph. *A. butzleri* could not be benefited by the co-culture at either condition. This might be due to the fact that as the rapid growth of *A. butzleri* is most likely dependent upon the release of some factors by *Acanthamoeba* but since in nutrient deprived NS the *Acanthamoeba* cells rounded off and started to encyst so probably no or very little factors could be released resulting in no noticeable growth of *A. butzleri* under any condition. The data represents mean±SE of two independent experiments.

3.3.8.4.2. Effect of *Acanthamoeba* on *A. butzleri* in VD

A. butzleri controls (without *Acanthamoeba*) showed massive growth as VD is the normal growth media for *A. butzleri*. *A. butzleri* in co-culture showed a significantly higher growth rate under both aerophilic and microaerophilic conditions at 22ºC (Figure 3.59). Similar effect was also observed in case of 30ºC but only by 24h post-co-culture. This might probably because of the fact that the unfavourable conditions (VD media and higher temperature) cause significant rounding off and encystation of *Acanthamoeba* trophozoites leaving them incapable of producing any further factors that might be supportive for enhanced growth of *A. butzleri*.

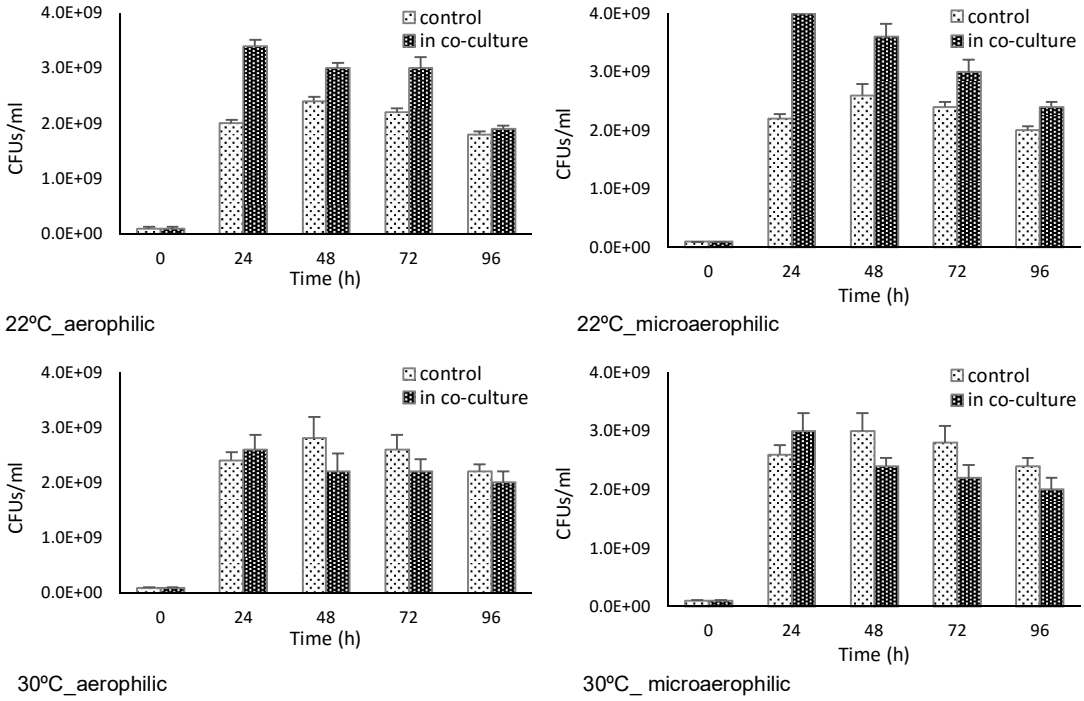


Figure 3.59: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1 in **VD** on *A. butzleri* ED-1. The co-culture was established under different growth conditions as mentioned under each graph. *A. butzleri* were significantly benefited by the co-culture at 22ºC by 72h and by 24h in 30ºC. This might be due to the fact that most likely the rapid growth of *A. butzleri* is dependent upon the release of some factors by *Acanthamoeba* but since at 30ºC the *Acanthamoeba* cells round off and start to encyst so probably more factor could not be released resulting in lesser growth of *A. butzleri* compared to 22ºC. The data represents mean±SE of two independent experiments.

3.3.8.4.3. Effect of *Acanthamoeba* on *A. butzleri* in AX2+

The effect of *Acanthamoeba* on *A. butzleri* was clearly demonstrated by co-culture findings in AX2+. At both the 22ºC and 30ºC as well as under both aerophilic and microaerophilic conditions, *A. butzleri* showed a significantly higher growth in co-cultures with *Acanthamoeba* as compared to solo growth in AX2+ even by 96h post co-culture (Figure 3.60). This observation clearly depicts the strong effect of *Acanthamoeba* factors releaed in the media on the massive growth of *A. butzleri*.

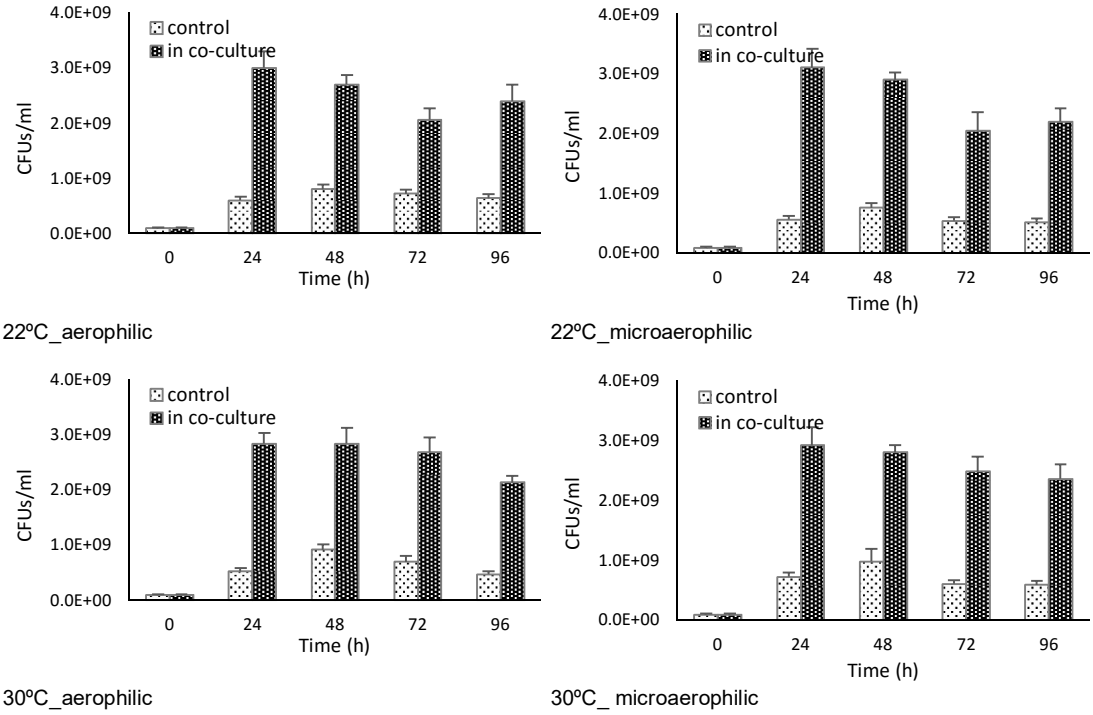


Figure 3.60: Effect of co-culture of *A. castellanii* Neff and *A. butzleri* ED-1in AX2+ on *A. butzleri* ED-1. The co-culture was established under different growth conditions as mentioned under each graph. *A. butzleri* were significantly benefited by the co-culture under all the conditions especially at 30ºC. This is most likely due to some factors released by *Acanthamoeba* in the media which not only had huge impact on the growth of *A. butzleri* but also on their longer survival. The data represents mean±SE of two independent experiments.

3.3.9. Effect of *A. butzleri* conditioned media (*Ab*CM) on excystment and encystment of *Acanthamoeba*

The effect of *A. butzleri* conditioned media (*Ab*CM) on the excystment and encystment of *Acanthamoeba* was studied over a period of four weeks. Filtered *Ab*CM was used to test the effect on excystment followed by encystment of *A. castellanii* Neff. A number of media were used as controls including AX2+, VD, (AX2+)+VD and (AX2+)+*Ab*CM (or sup VD-ED). The experiment was started with an equal number of cysts (5×10^5/mL) in these media separately. The effect was measured by daily observation of the suspensions and counting the cysts and trophozoites. Graphs were plotted separately for the number of trophozoites and cysts.

3.3.9.1. Effect of *Ab*CM on cysts pattern following excystment

After the addition of various media to the cysts, the excystment process started. The earliest excystment was observed in case of AX2+ media while the *Ab*CM took the longest time to attain the maximum excystment (by day-5) and was comparable with that of VD indicating the lack of any supportive factors in the *A. butzleri*-grown *Ab*CM (Figure 3.61)

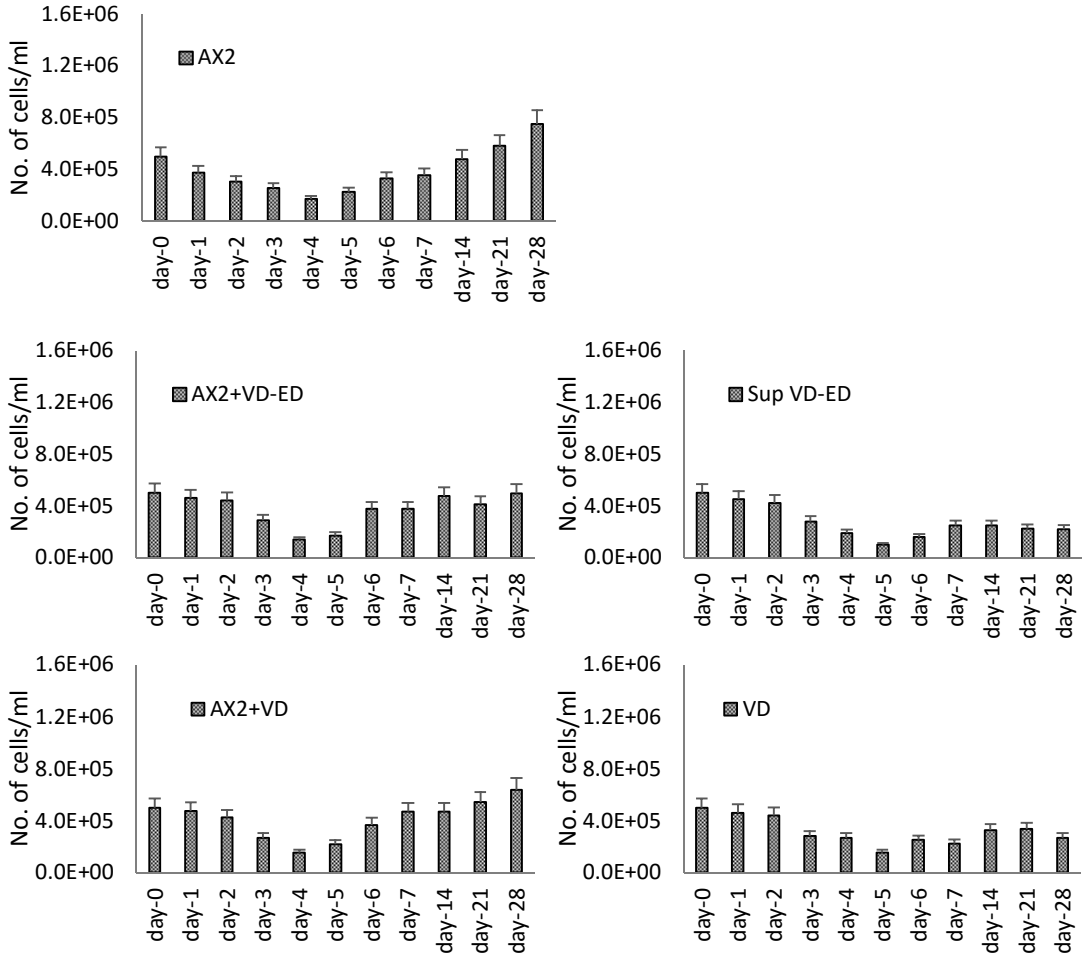


Figure 3.61: Cysts counts following excystment of *Acanthamoeba* cysts in different media. The main objective was to determine the effect of *Ab*CM (sup VD-ED) on the excystment while the other media and combinations of media were used as control to rule out any effect solely due to the media instead of *A. butzleri*-grown media. *Ab*CM did not seem to have a positive effect on early and rapid excystment (rather somewhat inhibitory effect) as better and early excystment was observed in case of AX2+ followed by (AX2+)+VD. *Ab*CM not only had least effect on excystment but also the encystment following excystment was rapid.

3.3.9.2. Effect of *Ab*CM on trophozoites pattern following excystment

*Ab*CM appeared to have the least effect on the excystment of *Acanthamoeba*. Compared to the results of AX2+ which had the earliest excystment by 24h, trophozoites could only be detected appreciably in *Ab*CM by day-3. Furthermore, the *Ab*CM media could no longer support the trophozoites which started to encyst again after day-5 and by second week most of the trophozoites had encysted again (Figure 3.62). These results were slightly lower than the ones observed in VD only. This indicates that the *Ab*CM does not have a supportive effect on the excystment of *Acanthamoeba* and whatever the excystment was observed was due to the VD media and not because of any factors released in the *Ab*CM by *A. butzleri*.

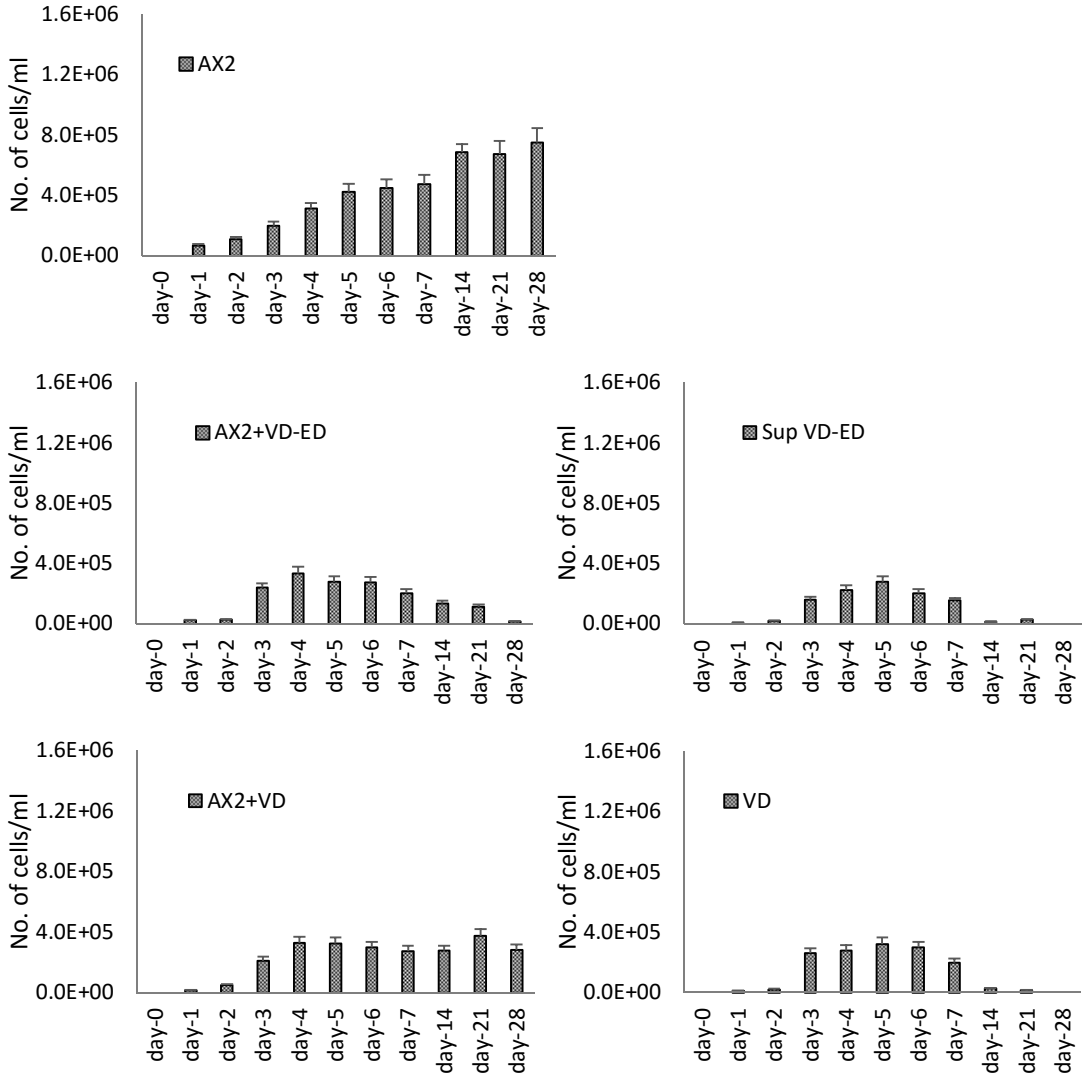


Figure 3.62: Trophozoites counts following excystment of *Acanthamoeba* cysts in different media. The main objective was to determine the effect of *Ab*CM (sup VD-ED) on the excystment while the other media and combinations of media were used as control to rule out any effect solely due to the media instead of *A. butzleri*-grown media. AbCM did not seem to have a positive effect (rather somewhat inhibitory effect) on early and rapid excystment as better and early excystment was observed in case of AX2+ followed by (AX2+)+VD. *Ab*CM not only had least effect on excystment but also the encystment following excystment was rapid.

If we look at the overall picture taking into account all the cells (trophozoites and cysts) as shown in Figure 3.63, it is interesting to find that cysts in AX2+ excyst and the trophozoite number increases constantly and overall there is a growing trend by the fourth week. While *Ab*CM shows a declining trend which is slightly lower than the declining trend in VD media.

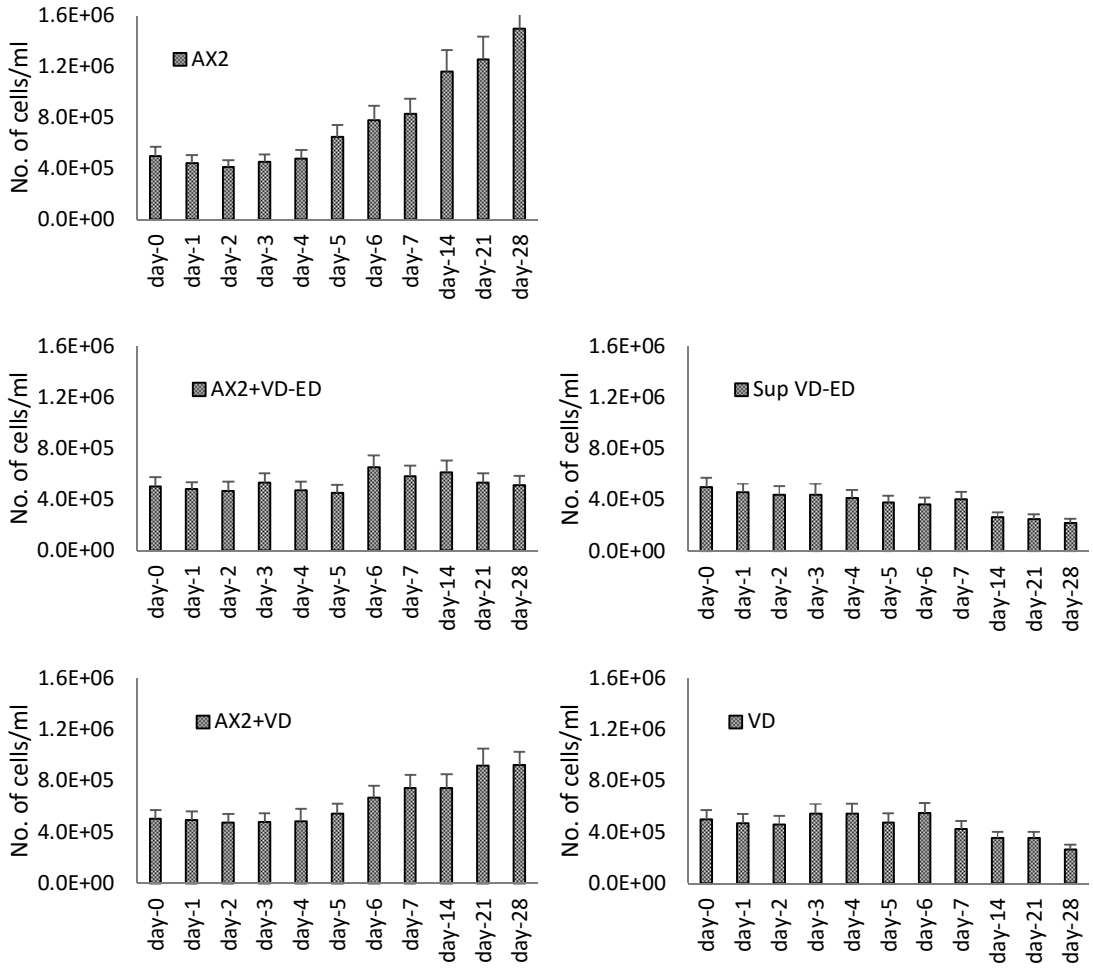


Figure 3.63: Total cell count (trophozoites+cysts) following excystment of *Acanthamoeba* cysts in different media. The main objective was to determine the effect of *Ab*CM (sup VD-ED) on the excystment while the other media and combinations of media were used as control to rule out any effect solely due to the media instead of *A. butzleri*-grown media. *Ab*CM did not seem to have a positive effect on net excystment and growth of cells as compared to the significantly better growth in AX2+ media.

Based on these findings it can be concluded that *A. butzleri* do not release any factors in the growth media that can be supportive for improved excystment of *Acanthamoeba* cysts or support their growth after excystment. These observations add to the findings that *A. butzleri* don't support growth of *Acanthamoeba* trophozoites (section 3.3.8.3.). Therefore, overall these findings indicate that *Acanthamoeba* are not the beneficiary during the periods of co-existence with *A. butzleri* while the reverse is not true and *A. butzleri* do take advantage of mutual interaction both extracellularly (from the factors released by *Acanthamoeba* (section 3.3.8.4.)) as well as intracellularly (by seeking protection and exploiting them as environmental reservoirs (sections 3.3.4./.5/.6)). Thus *A. butzleri* have great support in the environment from *Acanthamoeba* which they apparently seem to use to exploit *Acanthamoeba* themselves and under the

intracellular survival pressure of these predators have brighter chances of developing and improving their pathogenic skills which may pose a threat for human health.

3.3.10. Chemotactic attraction of *Acanthamoeba* towards *A. butzleri*

The chemotactic response of *Acanthamoeba* towards *A. butzleri* was studied under various conditions. The best concentration of agar, for the preparation of plates to be used for the chemotaxis experiment was found to be 2% as the 0.5% agar was too soft to handle while the 3% agar was too hard to work with (data not shown). Different cell movement patterns were observed as revealed by time lapse photography. Although most of the cells had directional movements towards the attractant, some cells showed circular motion or even movement in opposite direction before moving towards the attractant (Figure 3.64).

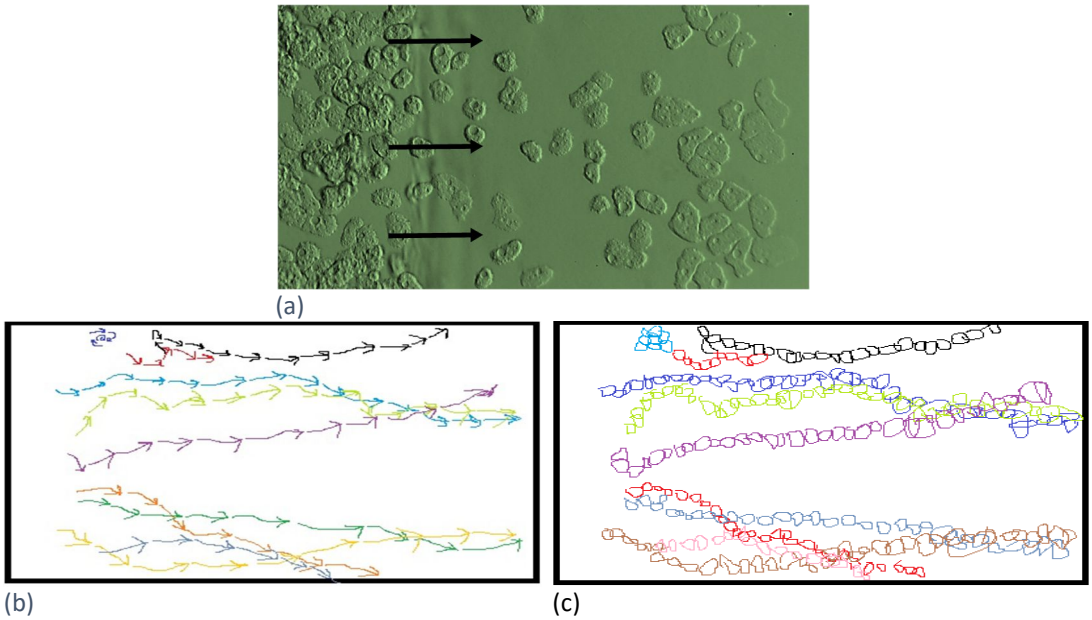


Figure 3.64: Chemotactic movements of *Acanthamoeba*. (a) The movement of *Acanthamoeba* cells (Arc-NB03) towards the chemo-attractant (*A. butzleri* Arco-L supernatant) showing the direction of movement of cells. (b) Movements of individual *Acanthamoeba* cells during their journey towards the chemo-attractant as recorded by time lapse photography. Individual cells are represented by different colours. Different behaviours of cells were seen; while most were found to move towards the attractant, some moved in circles or in opposite direction before moving towards the attractant. (c) Changes in the cell morphology during the course were also sketched.

The four combinations of *Acanthamoeba* and *A. butzleri* used for the following three different experimental conditions included Neff+ED-1, Neff+Arco-L, Arc-NB03+ED-1 and Arc-NB03+Arco-L, in NS or cell supernatant (heated or non-heated).

3.3.10.3.1. Chemotaxis in suspension (saline)

In case of A. castellanii Neff in **NS**, the chemotactic attraction towards both the *A. butzleri* strains (ED-1 and Arco-L) was slightly lesser than that towards control (NS) although a statistical difference between the control and the test group was not observed. With the other *Acanthamoeba* isolate, Arc-NB03, this trend was reverse i.e. amoebae showed slightly more attraction towards *A. butzleri* strains although there was not significant statistical difference (Figure 3.65). It is, therefore, apparent from these findings that the tested *Acanthamoeba* strains show no chemotaxis towards the *A. butzleri* strains while in NS.

3.3.10.3.2. Chemotaxis in cell supernatant

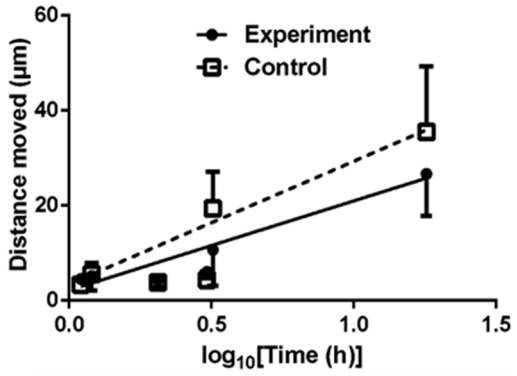
The response of Neff strain and the other *Acanthamoeba* soil isolates (Arc-NB03) towards the *A. butzleri* cell supernatant (*A. butzleri*-grown VD media, filtered) was studied. The cell supernatant was used directly without heating or heated (to denature any active factor involved).

3.3.10.3.2.1. Non-heated supernatant

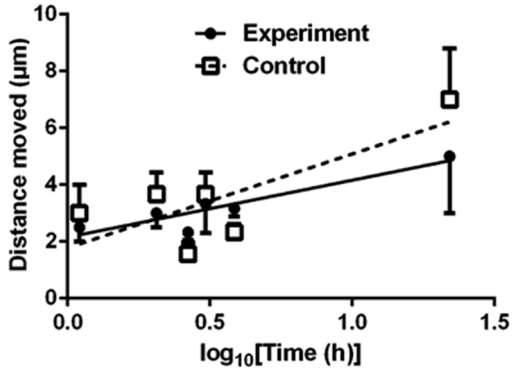
The Neff strain did not show significant attraction towards either of the *A. butzleri* strains although unlike the bacterial cells suspension case in NS, there was stronger attraction of amoebae towards the supernatant (Figure 3.66). The isolate (Arc-NB03), however, showed significant chemotactic attraction towards the *A. butzleri*-grown supernatant of Arco-L strain only (p=0.002395 at 95% confidence level).

3.3.10.3.2.2. Heated supernatant

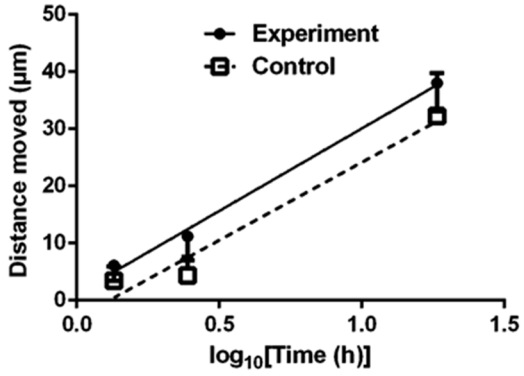
Similar results were observed in case of heat-inactivated *A. butzleri* supernatant as the Neff strain did not show chemotaxis towards either of *A. butzleri* isolates, while Arc-NB03 isolate showed significantly more attraction towards the inactivated supernatant of Arco-L strain only with the p value of 0.01554 at 95% confidence level (Figure 3.67). However, the p-value for non-heated supernatant was ~6.5 times lower than in case of heated supernatant. This indicates that most likely both heat-labile and heat-resistant chemotactic factors produced by *A. butzleri* Arco-L are involved in attracting *Acanthamoeba* (Arc-NB03 isolate).



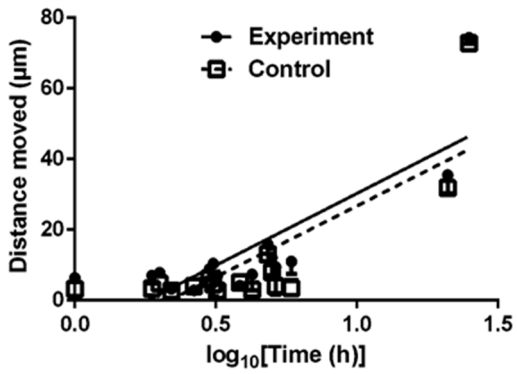
(a)
Neff (T4): **ED-1** suspension: NS
mean±SD, n=5
Exp: Y = 1.2286X + 4.7727
Ctrl: Y = 1.5916X + 7.6546
p = 0.07179



(b)
Neff (T4): **Arco-L** suspension: NS
mean±SD, n=4
Exp: Y = 2.0139X + 1.1417
Ctrl: Y = 0.901X + 5.4408
p = 0.6125



(c)
Arc-NB03 (T2): **ED-1** suspension: NS
mean±SD, n=3
Exp: Y = 1.7898X + 5.2829
Ctrl: Y = 1.7225X + 0.5662
p = 0.5692



(d)
Arc-NB03 (T2): **Arco-L** suspension: NS
mean±SD, n=5
Exp: Y = 2.4274X - 0.0955
Ctrl: Y = 2.448X – 3.5567
p = 0.9208

Figure 3.65: Graphical representation of the chemotactic response of *Acanthamoeba* Neff (T4) and Arc-NB03 (T2) towards *A. butzleri* ED-1 and Arco-L (in **suspension**). Four combinations were tested i.e. *A. butzleri* ED-1 with *A. castellanii* Neff (a) & Arc-NB03 (c) and *A. butzleri* Arco-L with *A. castellanii* Neff (b) & Arc-NB03 (d). The experiment was performed in agar plate having three troughs cut out with *Acanthamoeba* in centre and *A. butzleri* suspension and NS (control) each on either side trough. The distance travelled by *Acanthamoeba* on either side was measured after different intervals of time and a plot was drawn between the distance and the time (in log form for better representation) using Microsoft Excel showing the trend lines for each of the experiment and control groups. Statistical difference between the equations of lines was assessed by using GraphPad Prism 6 software. Statistical difference between the control and the experiment was calculated at 95% confidence level. [NS=Neff's saline; VD=Vandamme media; Exp=Experimental sample; Ctrl=Control sample]. (Shared work with another colleague in the laboratory).

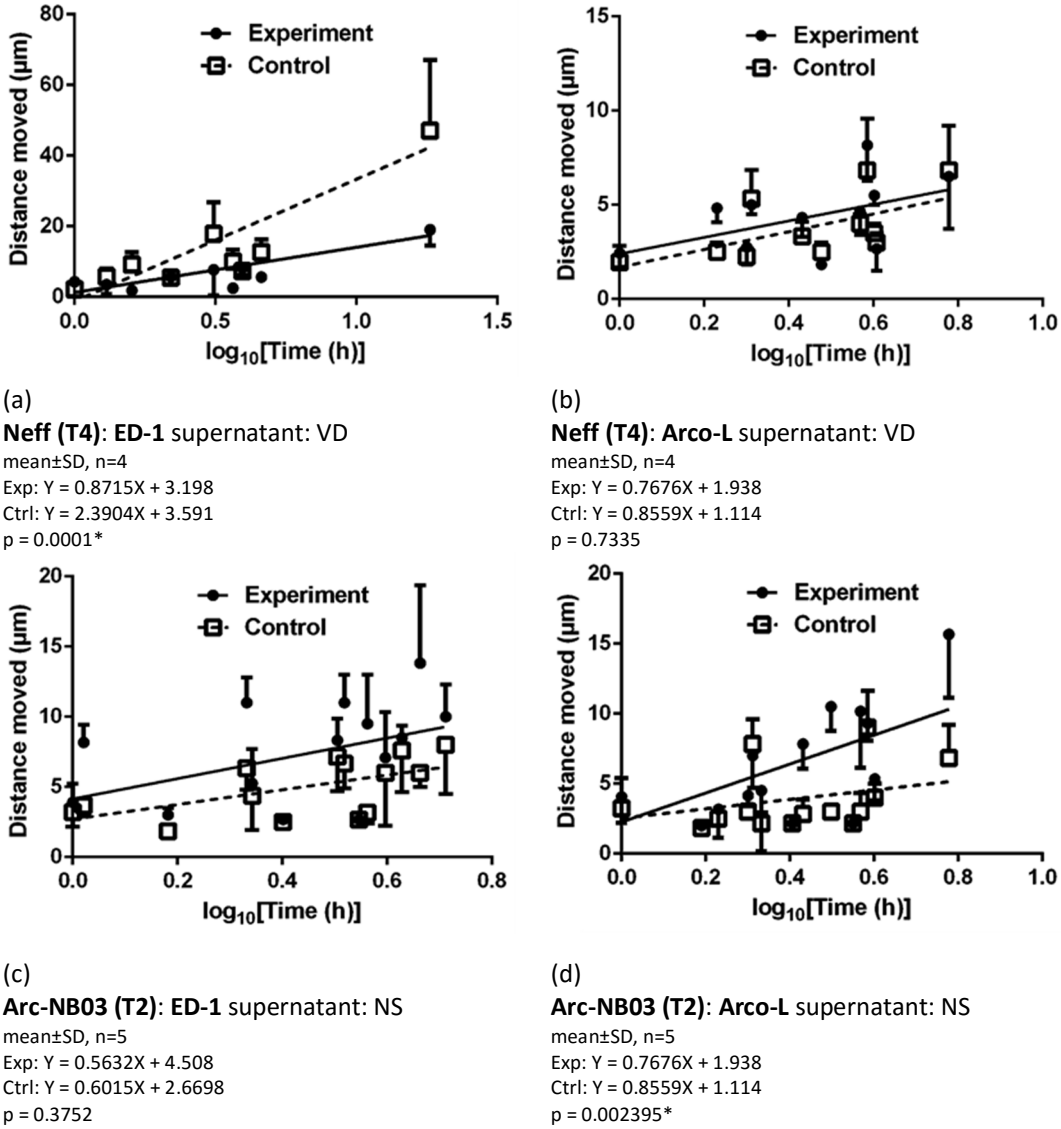
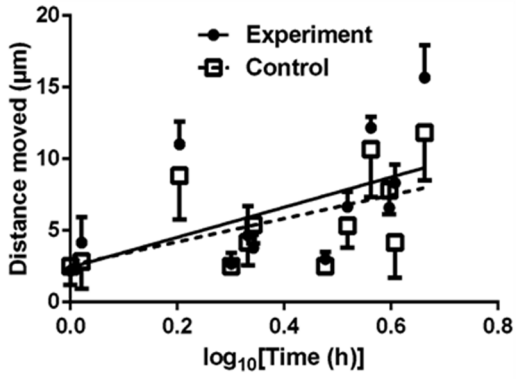
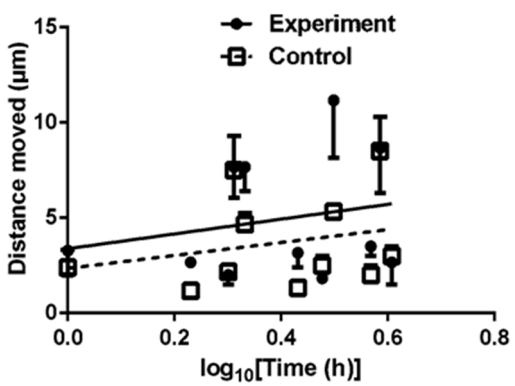


Figure 3.66: Graphical representation of the chemotactic response of *Acanthamoeba* Neff (T4) and Arc-NB03 (T2) towards *A. butzleri* ED-1 and Arco-L bacterial-grown media (**supernatant, non-heated**). Four combinations were tested i.e. *A. butzleri* ED-1 with *A. castellanii* Neff (a) & Arc-NB03 (c) and *A. butzleri* Arco-L with *A. castellanii* Neff (b) & Arc-NB03 (d). The experiment was performed in agar plate having three troughs cut out with *Acanthamoeba* in centre and *A. butzleri* supernatant (bacteria-grown media) and VD (control) each on either side trough. The distance travelled by *Acanthamoeba* on either side was measured after different intervals of time and a plot was drawn between the distance and the time (in log form for better representation) using Microsoft Excel showing the trend lines for each of the experiment and control groups. Statistical difference between the equations of lines was assessed by using GraphPad Prism 6 software. Asterisk (*) represents significance difference between the control and the experiment at 95% confidence level. [NS=Neff's saline; VD=Vandamme media; Exp=Experimental sample; Ctrl=Control sample]. (Shared work with another colleague in the laboratory).



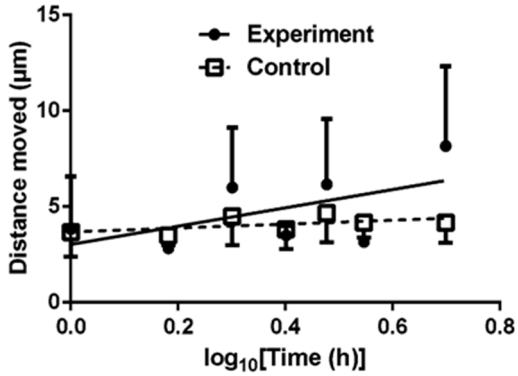
(a)

Neff (T4): ED-1 supernatant: VD
mean±SD, n=4
Exp: Y = 2.191X + 0.506
Ctrl: Y = 1.6441X + 1.234
p = 0.3094



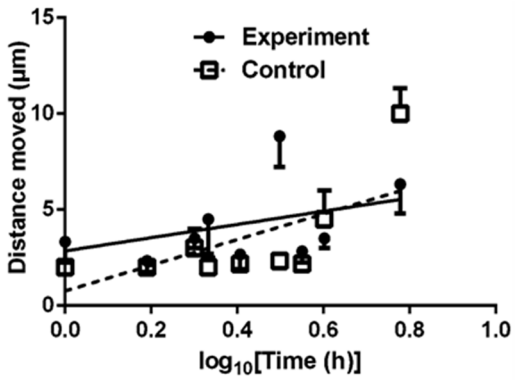
(b)

Neff (T4): Arco-L supernatant: VD
mean±SD, n=4
Exp: Y = 0.7167X + 2.951
Ctrl: Y = 0.6842X + 1.837
p = 0.9565



(c)

Arc-NB03 (T2): ED-1 supernatant: NS
mean±SD, n=2
Exp: Y = 0.9386X + 2.321
Ctrl: Y = 0.1458X + 3.685
p = 0.1241



(d)

Arc-NB03 (T2): Arco-L supernatant: NS
mean±SD, n=53
Exp: Y = 0.5927X + 2.5193
Ctrl: Y = 1.3667X – 0.4666
p = 0.01554*

Figure 3.67: Graphical representation of the chemotactic response of *Acanthamoeba* Neff (T4) and Arc-NB03 (T2) towards *A. butzleri* ED-1 and Arco-L bacterial-grown media (**supernatant, heated**). Four combinations were tested i.e. *A. butzleri* ED-1 with *A. castellanii* Neff (a) & Arc-NB03 (c) and *A. butzleri* Arco-L with *A. castellanii* Neff (b) & Arc-NB03 (d). The experiment was performed in agar plate having three troughs cut out with *Acanthamoeba* in centre and *A. butzleri* supernatant (bacteria-grown media) and VD (control) each on either side trough. The distance travelled by *Acanthamoeba* on either side was measured after different intervals of time and a plot was drawn between the distance and the time (in log form for better representation) using Microsoft Excel showing the trend lines for each of the experiment and control groups.. Asterisk (*) represents significance difference between the control and the experiment at 95% confidence level. [NS=Neff's saline; VD=Vandamme media; Exp=Experimental sample; Ctrl=Control sample]. (Shared work with another colleague in the laboratory).

Findings from the chemotaxis experiments indicate that *A. butzleri* release some chemotactic factors that attracts *Acanthamoeba* towards them making it easier for the amoebae to locate and access them although this property is not consistent between different strains of *Acanthamoeba* as indicated by strong response from the Arc-NB03 and the lack of effect from Neff strain. The demonstration of chemotaxis by Arc-NB03 towards both the heated and non-heated *A. butzleri* Arco-L supernatant also indicates that the factor involved in chemotaxis of these amoebae is possibly heat sensitive as the effect of the factor as chemo-attractant for *Acanthamoeba* was reduced by heating. However, further investigation would be required to isolate and identify the factor involved in chemotaxis.

Overall, the results of this chapter explore the effect of *Acanthamoeba* on bacteria taking the example of interaction between the emerging human pathogen *A. butzleri* with *Acanthamoeba*. Based upon the ease and versatility of this relation, *Acanthamoeba* do seem to act as environmental reservoirs for these bacteria. *A. butzleri* are prone to be easily located by *Acanthamoeba* through chemotactic attraction and are firmly attached on the cell membrane in the form of a pile (cap) with quick internalization although phagocytosis of *A. butzleri* appears to be a complex process requiring involvement of unevenly scattered sugar receptors on the surface of *Acanthamoeba*, actin polymerization and intracellular signalling pathways involving PI3-kinases, PTPs and vATPases.

The intracellular survival of *A. butzleri* in *Acanthamoeba* seems directly related to combating the killing strategies of the latter such as inhibiting lysosomal enzymes, preventing lysosome-phagosome fusion, avoiding phagosome acidification and impacting on the intracellular transport system. *A. butzleri* can exploit amoebae as environmental reservoir not only for survival but also to improve their pathogenic potential as manifested by their ability for limited proliferation/lysis and long term intracellular survival for upto two weeks normally or even upto 42 days as a results of enhanced pathogenic potential conferred by the repeated passages through amoeba cells. This strengthens the previously acknowledged role of amoebae in selection of pathogenic traits of bacteria.

Furthermore, *A. butzleri* under altered environmental conditions (presence of nicotinic acid) are shown to have the ability to change their pathogenic traits towards *Acanthamoeba* and thus better exploit amoebae as biological reservoirs with improved survival. *Acanthamoeba* also support extracellular survival (but not excystment) of *A. butzleri* by releasing growth supporting factors although they are not paid back by *A. butzleri* in the same way.

It is clear from these findings that *A. butzleri* are facilitated by *Acanthamoeba* in a variety of different ways. Therefore, *Acanthamoeba* seem to be potential environmental reservoirs and breeding grounds for the emerging human pathogens-*A. butzleri*. This can have direct consequences on human health and needs special consideration while designing any further treatment and control strategies.

3.4. Discussion

Free-living amoebae including *Acanthamoeba*, are widespread in nature and have been isolated from a wide variety of environmental matrices (Sandstrom *et al*., 2011). They constitute the main bacterial consumers in the environment and thus contribute to nutrients recycling and maintain the microbial populations (Greub and Raoult, 2004). The interaction of *Acanthamoeba* with bacteria is quite frequently due to co-existence of both organisms, widely throughout the environment (Schuster and Visvesvara, 2004). This opportunity for close contact leads to various levels of interactions between these organisms other than mere predator-prey relation.

Bacteria can resist the killing mechanisms of *Acanthamoeba* and thereby exploit amoebae as environmental reservoirs where they seek protection and their pathogenic traits are enhanced (King *et al*., 1988). This poses a health hazard for humans who are evolutionarily the dead end (Adiba *et al*., 2010). The role of *Acanthamoeba* as a reservoir for the environmental bacteria is of greater significance and, therefore, it has recently been focused intensively (Barker and Brown, 1994; Greub and Raoult, 2004; Marciano-Cabral, 2004a; Marciano-Cabral, 2004b; Tezcan-Merdol *et al*., 2004; Weekers *et al*., 1993). Although a number of bacteria have been shown to be capable of survival in amoebae the most thoroughly investigated interaction between *Acanthamoeba* and bacteria is for *Legionella pneumophila.*

It is strongly speculated that *Acanthamoeba* have acted as a "training ground" for *L. pneumophila* in acquiring pathogenic traits that enable them to survive the hostile environment of macrophages (Barker and Brown, 1994). This revolutionary finding gave rise to the concept that protozoans like *Acanthamoeba* play a vital role in the evolution of pathogenic environmental bacteria and, therefore, protists are the "missing link" between ecology and pathology (Barker and Brown, 1994; King *et al*., 1988; Ly and Muller, 1990).

Arcobacter constitutes a diverse group of bacteria having 18 different reported species (Cardoen *et al*., 2009; Collado and Figueras, 2011; Ferreira *et al*., 2015).

Among these, *Arcobacter butzleri* are most significant and they have been regarded as potential emerging zoonotic pathogens (Cardoen *et al*., 2009; Collado and Figueras, 2011). They are mainly responsible for enteritis (Arguello *et al*., 2015; Lau *et al*., 2002). Cases of *A. butzleri* infection have been reported from various parts of the world such as diarrhoeal stool samples of children in Thailand (Taylor *et al*., 1991), bacteraemia cases in Taiwan and Hong Kong (Lau *et al*., 2002; Yan *et al*., 2000), children in South African (Lastovica and Allos, 2008), bacteraemia cases in the United Kingdom (On *et al*., 1995), diarrhoea cases in Germany (Lerner *et al*., 1994). In another study by a surveillance network of *Campylobacter* infections in France, 29 *A. butzleri* infections cases were identified (Prouzet-Mauleon *et al*., 2006). An outbreak of *A. butzleri* was also reported in an Italian school (Vandamme *et al*., 1992).

Since *A. butzleri* are directly excreted out, therefore, they are frequently found in the environment (Collado and Figueras, 2011). Presence of amoebae (biological reservoirs) and human pathogens, like *A. butzleri*, in the same environment may pose a serious threat and requires a detailed investigation to understand the possible outcomes of this interaction. However, this has not been studied thoroughly although discrete work has been reported which indicates their transit association with *Acanthamoeba* and the uptake of bacteria in the cell (Fernandez *et al*., 2012; Medina *et al*., 2014). Therefore, the aim of this study is to explore the various dimension of *Acanthamoeba-A. butzleri* association.

It could be concluded from the results of current study that *A. butzleri* are capable of infecting *Acanthamoeba* cells *in vitro* and can also survive and multiply intracellularly. This conclusion was based on several observations as discussed below.

3.4.1. Interaction of *Arcobacter* with *Acanthamoeba-a*ttachment, internalization and survival of *A. butzleri* in *Acanthamoeba*

3.4.1.1. The capping phenomenon

A specific pattern of attachment with the *Acanthamoeba* cell in the form of aggregates on the surface of the cell, so-called “capping” phenomenon was found

whereby bacteria attached in the form of a cap instead of interacting all over (Figure 3.11). Bacteria could be seen attaching the surface of the cells almost immediately after the infection which indicates a rapid interaction between these organisms. A similar process of capping with *Acanthamoeba* has also been observed for some other bacteria like *Campylobacter jejuni* (Axelsson-Olsson *et al*., 2005), *Pseudomonas aeruginosa* (Bottone *et al*., 1994).

Capping has most frequently been observed with polar flagellate bacteria such as *C. jejuni* (Axelsson-Olsson *et al*., 2005), *P. aerginosa*, *Xanthomonas maltophilia* (Bottone *et al*., 1994) and *A. butzleri* (this study). However, capping has neither been observed for peritrichously flagellate bacteria like *E. coli* nor it has been seen in the non-motile bacteria like *Staphylococcus aureus* and *S. epidermidis* (Bottone *et al*., 1994). Although with the availability of data for more bacteria the situation will be much clearer with the current knowledge, it is reasonable to assume that the pattern of motility of bacteria may have something to do with their capping mode of infecting amoebae.

Another interesting observation is that the internalization of *C. jejuni*, *P. aerginosa* and *X. maltophilia* was much quicker than that of *E. coli*, *S. aureus* and *S. epidermidis* in which case it took much longer to engulf the bacteria despite the fact that they readily bound to the surface of amoeba (Bottone *et al*., 1994). This might be linked to the absence of capping as suggested by Bottone *et al*., (1994) as well. In the current study, the attachment (capping) and internalization of *A. butzleri* was very quick and it took bacteria only a few minutes to first appear inside the amoeba cells. In this regard, *A. butzleri* seem to be preferred food for *Acanthamoeba* than *E. coli*. This seems to be strengthens by the findings of Bottone *et al*., (1994) who concluded that "the suitability of a bacterial food source for *Acanthamoeba* spp. is intimately associated with the proclivity with which the bacteria bind to the trophozoite (and cyst) surface and the time course over which the bacterial substrate is internalised".

Therefore, in case of *A. butzleri*, this can be of greater significance in the real life situation in the environment. As observed in Chapter 2 (section 2.3.3.3.) the presence

of *Arcobacter* as the sole food source for *Acanthamoeba*, can have a significant effect on the recovery of genotypes and ultimately, in broader prospective, on the surveillance studies for *Acanthamoeba*. Moreover, in clinical conditions like AK and GAE, the recovery of all the genotypes associated with such conditions may be restricted if only one type of bacteria (*E. coli*) are used.

Cells were also found to use phagocytosis to engulf the bacteria (section 3.3.3.2.), a feature of amoeba commonly used for ingesting bacteria (Akya *et al*., 2009; Allen and Dawidowicz, 1990; Alsam *et al*., 2005; Chambers and Thompson, 1976; Medina *et al*., 2014). Transmission electron microscopy also indicated the presence of a large number of mitochondria in close proximity of the internalized bacteria in phagosomes (Figure 3.13). Although the reason for the close association of mitochondria and phagosome was not further investigated but this phenomenon has already been observed. Horwitz (1983) was the first to report the presence of smooth vesicles and mitochondria around the phagosome containing ingested *L. pneumophila* although he could not explain it. Later, Tilney *et al*. (2001) further investigated this and found physical connections between the endoplasmic reticumum vesicles, mitochondria and the phagosome containing *L. pneumophila*. It was found the thickness of the infected phagosome changes to match that of endoplasmic reticulum which then leads to the attachment of ribosomes to the membrane of phagosome. The membrane conversion was found to be a four-stage process which is used by *L. pneumophila* for intracellular survival.

Internalization of *A. butzleri* was found to be quick and the beating bacteria could be seen inside the cells in vacuoles approximately 10 min after the infection while the lysis of cells can be observed as early as 45 min (Figure 3.15). This was in contrary to *C. jejuni* infection of *Acanthamoeba* where these bacteria were seen inside the cell 1h after the infection (Axelsson-Olsson *et al*., 2005). It is interesting that among the population of *Acanthamoeba* not all the cells are equally infected (Figure 3.28). Moreover, the pathogenic behaviour of these bacteria towards *Acanthamoeba* was seen more pronounced during the early stages of infection (~2h post-infection) (Figure 3.33 and 3.34).

3.4.1.2. Chemotactic response of *Acanthamoeba* towards *A. butzleri*

How easily *Acanthamoeba* can locate and approach *A. butzleri* was studied by undertaking chemotactic experiments for *Acanthamoeba* (Neff and Arc-NB03-a soil isolate) and *A. butzleri* either using bacterial suspension in NS or by using *A. butzleri*-grown media (supernatant). The Neff strain did not show any significant attraction towards either of the *A. butzleri* strains neither when bacteria suspension was used nor when cell supernatant was used. But the Arc-NB03 isolate showed a much stronger attraction towards the Arco-L strain (p=0.0023 at 95% confidence level) (Figure 3.65, 3.66, 3.67). This indicates, that *A. butzleri* have differential attraction by different isolates of *Acanthamoeba* ranging from strong to no attraction. It might also be related to T type of *Acanthamoeba* used as Neff is a T4 while Arc-NB03 is a T2 isolate.

These findings are important and reinforce the findings of Chapter 2 in the sense that use of just one type of bacteria should be discouraged as different bacteria have different chemo-attractive behaviour from different types of *Acanthamoeba*. This can have an impact on the *Acanthamoeba* T typing surveillance studies in environmental as well as in clinical samples. This observation, however, contradicts the findings of Schuster *et al*., (1993) who observed a positive chemotactic response shown by all the bacteria studied (*Bacillus cereus, Clostridium tetani, Chromobacter violaceum, Enterobacter cloacae, Klebsiella pneumonia, Micrococcus luteus, P. aeruginosa, P. fluorescens, Serratia marcescens, Staphylococcus aureus, Yersinia enterocolitica*).

It is strongly speculated, based on the findings of chemotaxis experiments, that *A. butzleri* release some chemotactic factors (only when they are grown in the growth media i.e. VD but not when they are in NS) that is differentially responded to by *Acanthamoeba* isolates. These factors are probably a combination of both heat-labile as well as heat-resistant substances because the heat treatment of *A. butzleri*-grown media caused ~6.5 times drop in the chemotactic attraction. However, further experiments with more types of *Acanthamoeba* are required specifically to explore further the relationship between *Acanthamoeba* and *A. butzleri* and association with different T types.

Overall, these results show that *A. butzleri* are strongly attracted by *Acanthamoeba*. It has already been observed in this Chapter that *A. butzleri* are readily attached and internalized by *Acanthamoeba* (section 3.3.3.1. and 3.3.3.2.). Together, all these findings indicate that *A. butzleri* appear to be a favourable food for *Acanthamoeba* as they are easily located by amoebae, are readily attached to the cells and get internalized into the cells within few minutes.

3.4.1.3. Role of sugars on uptake of *A. butzleri* by *Acanthamoeba*

The attachment and internalization of bacteria in cells is a complex phenomenon which requires the involvement of a number of factors and components from the both sides (Samrakandi *et al*., 2002). The importance of mannose receptors in uptake of yeast was demonstrated by (Allen and Dawidowicz, 1990). The importance of mannose binding protein is already well known for bacteria (Akya *et al*., 2009; Alsam *et al*., 2005; Declerck *et al*., 2007; Harb *et al*., 1998; Medina *et al*., 2014). Attachment inhibitory assays using glucose, galactose and mannose revealed the importance of these sugars in the attachment of *A. butzleri* to *Acanthamoeba*. In the current study, the role of sugars in the attachment of *A. butzleri* with *Acanthamoeba* was investigated in detail using not only just Neff (Figure 3.16) but also three other soil isolates of *Acanthamoeba* (Figure 3.17) using three different strains of *Arcobacter* (ED-1, Arco-L and RM-4018) to better understand the significance of sugars and whether the finding is just restricted to a particular strain of *Acanthamoeba* or *Arcobacter*.

Overall, all the three sugars seemed to play an important role in attachment, however, there was wide variation in the results for different combinations of *Acanthamoeba* and *A. butzleri* strains used. The most significant concentration of sugars to exert their impact was 100 mM while the 10 mM concentration had very little or no effect. For the Neff strain galactose seemed to have most significant role as compared to glucose and mannose for all the three strains of *Arcobacter*. This was in consistence with the observations of Aslam *et al*., (2005) and Medina *et al*., (2014) but inconsistent with the findings of Akya *et al*., (2009) where more bacteria (*Listeria monocytogenes*) were internalised in mannose-treated *Acanthamoeba* cells.

This indicates variation in the role of sugar receptors in the uptake of different bacteria. This was also evident in the current study as wide variation in the uptake of bacteria for different strains of *A. butzleri* and even for different strains of *Acanthamoeba* was found. However, overall mannose seemed to have most potent effect on attachment for the Eco strain of *Acanthamoeba* used. Similarly glucose had the most significant impact in case of Arc isolate. Moreover, overall the RM-4018 was least affected as compared to the ED-1 and Arco-L strains of *Arcobacter*. It is clear from these results that although sugars have effect on the uptake of *A. butzleri* to the *Acanthamoeba* but the effect is not consistent and varies widely among the different strains of *Acanthamoeba* as well as *Arcobacter*.

3.4.1.4. Role of actin polymerization and intracellular signalling pathways

The importance of actin polymerization and cytoskeleton rearrangement in phagocytosis has been investigated (Akya *et al*., 2009; Cossart and Sansonetti, 2004; Elliott and Winn, 1986). The role of various pathways and processes in the uptake and killing of *Arcobacter* by *Acanthamoeba* was studied by use of different inhibitors of phagocytosis. A number of mechanisms were found to be involved including microfilaments and cytoskeleton rearrangement which are normally dependent upon actin polymerization and PI3 kinase activity. Actin polymerization was found to be important in the uptake of *Arcobacter* as indicated by the lower uptake by the cells treated with cytochalasin D (actin polymerization inhibitor) which resulted in decreased uptake of bacteria (Figure 3.18). Cytochalasin D has been used for this purpose for different bacteria including *E. coli, L. monocytogenes, L. pneumophila* with similar findings (Alsam *et al*., 2005; Elliott and Winn, 1986; King *et al*., 1991; Moffat and Tompkins, 1992).

Actin polymerization, in turn, is regulated by phosphoinositide 3-kinases (PI3-K) which are also involved in a number of other activities as well. Wortmannin is an irreversible inhibitor of PI3-K. PI3K signalling pathway was found to be important in the uptake of *A. butzleri* by *Acanthamoeba* as demonstrated by the use of wortmannin (PI3K inhibitor) which resulted in decreased phagocytosis (Figure 3.19) as shown by Akya *et al*., (2009) as well.

Protein tyrosine phosphatases (PTPs) form a large group of enzymes that execute a vital role in both intra- and inter-cellular signalling. PTPs work antagonistically to protein tyrosine kinases (PTKs) for the regulation of signal transduction in cell. PTKs phosphorylate while PTPs de-phosphorylate proteins, thereby together they both regulate functions of proteins in response to the external signals received. The significance of the intracellular signalling pathway was also confirmed by the use of sodium orthovanadate (a tyrosine protein phosphatase inhibitor) which increased the bacterial uptake by the *Acanthamoeba* cells (Figure 3.20). This is consistent with other findings (Alsam *et al*., 2005; Medina *et al*., 2014).

3.4.1.5. Fate of *A. butzleri* in phago-lysosome

After the ingestion of many different kind of bacteria such as *Mycobacterium lepraemurium* and *Salmonella typhimurium* by phagocytic cells, the bacteria get enclosed in phagosomes. On the fusion of lysosomes these phagosomes convert into phagolysosomes. Certain chemical agents like ammonium chloride, have the ability to neutralize acidity and inhibit the normal fusion of lysosomes with phagosomes (Gordon *et al*., 1980; Hart *et al*., 1983; Young and D'Arcy Hart, 1986). The role of vacuolar acidity in killing of *A. butzleri* was also accessed by knocking down the effect of acidity in different ways. Direct effect of lysosomal low pH was accessed by using ammonium chloride (a weak base) which is taken up by the *Acanthamoeba* cells and accumulates in the acidic compartments (lysosomes) causing an increase in the pH thereby disrupting the normal operational environment of lysosomes . This was demonstrated by the elevated number of *A. butzleri* bacteria recovered after infection of pre-treated *Acanthamoeba* cells (Figure 3.22).

Monensin resulted in lesser uptake of bacteria as compared to the non-treated control cells in a concentration-dependent manner (Figure 3.23). Monensin is a cationic ionophore that affects receptor-mediated endocytosis by alkalinisation of lysosomal content (Maxfield, 1982). It increases the pH of the organelles like lysosomes by exchanging the H^+ with another cation like Na^+ and thereby increases the pH without affecting the overall polarity of the system. It also has an impact on the intra-cellular transport system. Monensin is lipophilic in nature so it is inserted into cellular

membranes and slows down intracellular transport of newly synthesised secretory proteins and other molecules while the main site of action is Golgi complex (Prabhananda and Kombrabail, 1992; Tartakoff, 1983). In the current study, reduction in uptake of bacteria by monensin-treated *Acanthamoeba* cells, indicates the role of phagolysosomal acidification and intracellular transport system in the killing of internalized *A. butzleri* by *Acanthamoeba*.

Bafilomycin A is primarily a macrolide antibiotic but it also acts as an inhibitor for v-ATPases which are involved in phagosome acidification. The v-ATPases are located in the cell membranes of many intra-cellular compartments including lysosomes, endosomes and secretory vesicles. These consist of two main domains, the V1 (extra-membrane) and V0 (intra-membrane) which are both actually comprised of different sub-domain components which included A, B, C, D, E, F, G and H in case of V1 while the V0 consists of a, c, c', c" and d. This constitutes a kind of proton pump that is driven by ATP. The H^+ are pumped from the cytoplasmic compartment into the lumen of the lysosomes causing a drop in pH (Forgac, 1999; Stevens and Forgac, 1997). Bafilomycin A blocks this system by attaching to the subunit c. Using the energy from ATP, the v-ATPases push the H^+ across the membrane into the lumen of lysosomes. Bafilomycin A blocks this process and thereby the lowering of lysosomal pH is halted. In the current study, as a result of inhibition of v-ATPases, the killing of *A. butzleri* was also prevented (Figure 3.24 and 3.25). This indicates a direct involvement of v-ATPases in intracellular killing of *A. butzleri* in *Acanthamoeba*. Similar findings were reported by Akya *et al*., (2009) for *L. monocytogenes* and *Acanthamoeba*.

Surmain is a polybasic anion and accumulates in the lysosomes where it interferes with the normal function of lysosmal enzymes. It inhibits a number of lysosomal enzymes including proteases (Gildea *et al*., 2005). It also prevents lysosome-phagosome fusion (Draper *et al*., 1979). Suramin resulted in increased uptake of *A. butzleri* stressing the role of early lysosome-phagosome fusion and the lysosomal enzymes in the killing of *A. butzleri* (Figure 3.26). Similar findings have been reported for *L. monocytogenes* and *Saccharomyces cerevisiae* (Sipka *et al*., 1991).

Overall, these results indicate that a number of factors and strategies are employed in the uptake of *A. butzleri* by the *Acanthamoeba* cells including the monosaccharide cell surface domains, actin polymerization, cytoskeletal rearrangements, PI3K signalling pathway and protein tyrosine phosphatase. Furthermore, the bacteria are killed as a result of phagosome-lysosome fusion by the action of lysosomal enzymes which require a low pH to accomplish this job. It can be concluded that similar strategies are used by the *Acanthamoeba* cells for uptake and killing of *A. butzleri* as commonly used by most other phagocytic cells.

3.4.2. Intracellular survival of *Arcobacter* in *Acanthamoeba*

Firstly, the infection assays were performed in suspension (planktonic mode) rather than in 24-well plate as it is usually performed (section 3.2.8.). This was practised to avoid any loss of infected cells due to detachment especially during washing of cells. In case of suspension infection, cell aliquots (1 mL) were taken out at intended time points. This ensured no loss of cells due to detachment. Suspension infection has been used to study the interaction of *Burkholderia pseudomallei* with *Acanthamoeba* sp. (Inglis *et al*., 2000).

At an MOI of 100:1, nearly 60% of the *Acanthamoeba* were infected after the addition of *Arcobacter* to the *Acanthamoeba* suspension and the ratio of lysed cells was quite low (~5%) (Figure 3.28). However, the infection rate was much higher than that of *L. pneumophila* (Moffat and Tompkins, 1992) that was only 4.4% with an MOI of 100:1. Infection of *Acanthamoeba* cells with different strains of *A. butzleri* revealed a good percentage of internalization (number of bacteria engulfed by *Acanthamoeba* cells out of the total number of bacteria added) ranging from 0.6% (for RM-4018) to 1.1% (for ED-1). *A. butzleri* showed proliferation for up to 2h after time zero of infection (Figure 3.33). Most of the lysis of cells was also observed during this period.

Legionella sp. has also been shown to proliferate in *A. castellanii* cells but this was found to follow a specific pattern whereby bacteria first decline for an initial few days of infection but start proliferating after that until around 20 days (Moffat and

Tompkins, 1992). Intracellular *A. butzleri* declined after 2h until 24h and then upto 48h (Figure 3.33). Further experiments with extended monitoring of intracellular bacteria beyond 48h indicated that the bacteria were not only completely eliminated by nearly two weeks after the initial infection but they survived at a very low number during this time before being completely wiped out by *Acanthamoeba*.

Chlamydia pneumonia have shown continued infection for upto 14 days (Essig *et al*., 1997). *C. jejuni* showed somewhat similar intracellular survival pattern as *A. butzleri*, however, they lacked the initial proliferation phase as in *A. butzleri* (Olofsson *et al*., 2013). *Mycobacterium* sp. also showed much longer survival in *Acanthamoeba* for upto 30 days of the monitored period (Berry *et al*., 2010).

The lysis of *Acanthamoeba* cells after infection with *A. butzleri* was found to be as a consequence of direct infection and not due to any factors produced by the bacteria (section 3.3.4.2.). The lysis of the cell follows a nearly uniform pattern which is initiated by the immobilization of the cell followed by loss of integrity of the cellular organelles and eventual rupture of the cell membrane leaking the cellular content and freeing the still live bacteria as well (Figure 3.30).

It can be concluded from these experiments that *A. butzleri* initially show a proactive pathogenic behaviour causing some lysis of *Acanthamoeba* cells but they cannot continue this for longer and are overpowered by the hostile environment of *Acanthamoeba* but they still have the potential to survive inside the cell probably under a symbiotic relationship at a very low number. This is a very versatile behaviour of a pathogen towards its predator.

These observations were also supported by the findings of the plaque assay for *Acanthamoeba* and *A. butzleri* where the cells were infected and then immobilized under the agar to look for any plaques formed due to the lysis of cells by the bacteria. Very few and small plaques were observed (Figure 3.35) which again confirms a limited pathogenic potential of these bacteria against *Acanthamoeba*. Plaque assay has been routinely used for studying the pathogenic potential of bacteria in terms of

spreading in a cell monolayer notably *L. monocytogenes* (Deshayes *et al*., 2012; Portnoy *et al*., 2002; Sun *et al*., 1990), however, it has not been used for this purpose in studying interaction of bacteria with *Acanthamoeba*.

3.4.3. Re-infection experiments with *A. butzleri* and *Acanthamoeba*

Intra-amoebal survival of bacteria is hypothesized as an important step in their training for adaptation to mammalian cells (Molmeret *et al*., 2005). In the current study, the re-infection experiments were performed to assess the effect of intracellular survival of *A. butzleri* in *Acanthamoeba* on their virulence towards *Acanthamoeba*. The amoeba cells were infected and the bacteria were recovered. The recovered bacteria were again used for a new infection. The process was continued for upto 15 re-infections. The results indicated a significant effect of recovered bacteria on enhanced internalization at time zero of infection as compared to the control bacteria (Figure 3.36, 3.37). The effect was significant for the initial six reinfections and it started to diminish in the later re-infections. This indicates that although the bacteria recovered from *Acanthamoeba* show more virulence towards the *Acanthamoeba* but continued use of these bacteria for infection and reinfection causes a decline in their virulence. The bacteria recovered from the *Acanthamoeba* seemed to have gross morphological changes in being smaller and more motile than the normal bacteria (Figure 3.37-b,c). The recovered bacteria were used for undertaking complete infections for extended periods of time to see the effect on the length of survival of the recovered bacteria in the *Acanthamoeba* cells (Figure 3.38). The control WT bacteria did not show survival inside *Acanthamoeba* beyond two weeks.

On the other hand, the recovered bacteria not only had better but also the longer survival in the *Acanthamoeba* cells. The longest survival period for the recovered bacteria up to 42 days post-infection. The fold-change of recovery of *A. butzleri* ED-1 for the recovered bacteria compared to the WT bacteria at each time point until day-7 for the 15 re-infections was calculated and compared (Figure 3.39). The fold change was calculated for each time point by dividing the number of bacteria recovered from re-infection by the WT bacteria recovered at the same time point

from control counterpart. From day-0 towards day-7, the difference increases because of the declining number of control bacteria as compared to the bacteria from re-infection which show a longer and better survival of bacteria although at low number.

The significance of amoebae in enhancing the virulence traits of bacteria was a revolutionary finding that changed the way the protozoa-bacteria relation was perceived. Cirillo *et al*., (1994) demonstrated that growth of *L. pneumophila* in environmental amoebae enhanced their ability to enter different types of cells including *A. castellanii* where 10-100 fold increase was observed as a result of intra-amoebal survival. Even with other cells similar effect was observed including human monocytes (100-1000 fold increase), epithelial cells (100-1000 fold increase) and murine macrophages (10-100 fold increase). Furthermore, Cirillo *et al*., (1999) demonstrated that the survival of *L. pneumophila* in *Acanthamoeba* enhanced their virulence and the strategies to gain entry into the monocytic cell. Moreover, growth in the lungs of mice was also increased. This was an important finding which suggested that *L. pneumophila* grown in amoebae present in domestic water supplies are responsible for the Legionnaires' disease in humans. This effect has even been observed in plant-associated bacteria. It has recently been reported that in case of *Mesorhizobium loti* bacteria that colonize legume roots, their growth inside in *Acanthamoeba* enhanced the ability to develop more nodules on *Lotus corniculatus* plant (Karas *et al*., 2015).

Overall, the results of re-infection experiments in the current study indicate that the survival of *A. butzleri* inside *Acanthamoeba* has a significant impact on their pathogenic potential towards *Acanthamoeba* in terms of better invasion capabilities and longer survival in *Acanthamoeba*. This is an important finding which may have an implicit implication under the natural environmental conditions where these predator prey are under frequent interactions. *Acanthamoeba* may serve as training grounds for these bacteria in improving the skills of these bacteria in adapting or even exploit the hostile cellular mechanisms of *Acanthamoeba* and these bacteria with enhanced pathogenicity may pose a threat to the humans and animals.

3.4.4. Sensing and responding the environmental changes by *A. butzleri* and possible role in survival in *Acanthamoeba*

How the environmental changes be detected and responded by *A. butzleri* in terms of their enhanced virulence can have an important effect on the survival adaptation of these bacteria in *Acanthamoeba*. The bacterial two-component system that helps bacteria in sensing the environmental changes has been observed to be activated by the pyridine derivative nicotinic acid (NA) for different bacteria. NA can modulate the gene expression of *L. pneumophila* and induce virulence traits (Edwards *et al*., 2013). *Bordetella pertussis* can change the expression of hundreds of genes and ultimately the virulence traits in response to the various concentrations of NA (Schneider and Parker, 1982). Similarly in *E. coli* the effect is manifested on motility and expression of certain proteins (Utsumi *et al*., 1994). In *B. pertussis* and *C. glabrata* NA is involved in regulation of virulence factors' expression (adhesions, toxins, and agglutinating factors) (Cotter and DiRita, 2000; McPheat *et al*., 1983; Schneider and Parker, 1982). In *B. pertussis,* the two-component system (BvgA/S) is modulated by NA (Cummings *et al*., 2006; Melton and Weiss, 1989). NA by inactivating the BvgA/S two-component system represses a wide variety of gene expression states and virulence factors (Cotter and DiRita, 2000; Cummings *et al*., 2006; McPheat *et al*., 1983; Schneider and Parker, 1982). The homology of the two-component system (LetA/S) of *L. pneumophila* with that of the BvgA/S system of *B. pertussis* (Edwards *et al*., 2010) drove attention towards the possible role of nicotinic in modulation of gene expression in the former as well. It was found that at 5 mM, NA tends to trigger various premature phenotypic traits including evasion of lysosomal degradation, motility, cytotoxicity toward macrophages and sodium sensitivity. At the gene level, a total of 213 genes were found to be altered by the NA pre-treatment although the most expressed was the one related to the protection against high concentrations of NA (Edwards *et al*., 2013).

The impact of NA on modulating *A. butzleri* two-component system and the subsequent induction of virulence traits has not been studied. This was a baseline study to assess the impact of activation of the two-component system of *A. butzleri* by NA and any subsequent activation of virulence trait. The results of the current

study with *A. butzleri* clearly demonstrated morphological changes in *A. butzleri*; bacteria being more active and smaller, the features which are usually seen with the more virulent nature of *A. butzleri*. Also, the NA-treated bacteria showed a more virulent behaviour towards the *Acanthamoeba* cells. This was manifested by the significantly higher rate of infecting *Acanthamoeba* cells than the control WT bacteria (Figure 3.41-b). Furthermore, the rate of internalization of bacteria into the *Acanthamoeba* cells at t=0 was significantly higher than the control WT bacteria (Figure 3.40). These findings signify the importance of activation of two-component system of *A. butzleri* by NA and its impact on the enhanced virulence traits towards *Acanthamoeba*. Further investigation on the genes expression profile of *A. butzleri* are required to precisely understand which genes are specifically up-regulated during this process. This may also be useful in understanding the role of various genes in the pathogenicity of *A. butzleri*.

3.4.5. Consequences of co-existence of *Acanthamoeba* and *A. butzleri*

The co-cultures of *Acanthamoeba* and *A. butzleri* were studied under a variety of environmental conditions including temperature, oxygen levels and culture media (NS, VD and AX2) to determine the effect on both the organisms. In **NS** under both aerophilic (Figure 3.46, 3.47) and microaerophilic (Figure 3.48, 3.49) conditions there was no proliferation of *Acanthamoeba* neither at 22ºC nor at 30ºC. This might indicate a lack of any substance produced by bacteria to support *Acanthamoeba* growth but since the bacteria were under nutrient deficiency stress so it may not be true at this level. However, de Moraes *et al*., (2008) in their co-culture study noticed that when *A. castellanii* trophozoites were transferred from nutrient medium to saline, cells retained their morphology for upto 7 days but started encysting thereafter. In the presence of *E. coli*, the trophozoites grew much better with the addition of 1×10^9 bacteria/mL. The proliferation of trophozoites in the saline was also observed in dose-dependent manner for other bacteria as well including *Micrococcus luteus*, *P. aeruginosa*, *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus* and *Enterobacter cloacae* for upto 2-5 days but there was no further increase in the number of trophozoites. On the other hand, under both the aeration conditions *A. butzleri* were also unable to be benefited by *Acanthamoeba*

and, therefore, their number declined continuously (Figure 3.58). However, it does not rule out lack of any substances released by *Acanthamoeba* to support the growth of bacteria as the *Acanthamoeba* were also under nutrient deficiency stress and encysted.

In **VD** under aerophilic conditions (Figure 3.50, 3.51), *Acanthamoeba* were able to grow although not as extensively as in AX2 but no growth was observed in co-culture which indicates a negative impact of *A. butzleri* on *Acanthamoeba*. Similar observations were made under microaerophilic conditions (Figure 3.52, 3.53) although the growth of *Acanthamoeba* was lesser in control groups due to microaerophilic conditions but there was a decline in the number of *Acanthamoeba* in co-cultures. This indicates again a negative effect of *A. butzleri* on *Acanthamoeba*. On the other hand *A. butzleri* had significantly better growth in VD under microaerophilic conditions and 22ºC followed by bacteria in VD under aerophilic conditions and 22ºC which appears to be due to more favourable microaerophilic conditions (Figure 3.59). These findings again indicate the positive impact of *Acanthamoeba* towards enhanced growth of *A. butzleri* in co-culture. It is interesting that at 30ºC under both aerophilic as well as microaerophilic conditions the growth of *A. butzleri* was lesser than that of the control solo cultures of *A. butzleri* except at t=24h. This was probably due to early encystation of *Acanthamoeba* at 30ºC which halted the production of the growth factors produced by *Acanthamoeba* trophozoites due to their rounding and encystation.

In **AX2**, *Acanthamoeba* had a very good growth in controls at 22ºC and aerophilic conditions (Figure 3.54, 3.55) and slightly less growth in 22ºC and microaerophilic conditions (Figure 3.56, 3.57), however, in the co-cultures in both the conditions the growth of *Acanthamoeba* was significantly lesser as compared to the controls. A similar trend was observed in case of 30ºC although the growth of *Acanthamoeba* controls was lower than that at 22ºC due to higher temperature. These findings also indicate that the presence of *A. butzleri* has a negative impact on the growth of *Acanthamoeba*. As far as the impact of *Acanthamoeba* on *A. butzleri* in concerned, *A. butzleri* had significantly better growth in co-cultures than in the solo cultures

(Figure 3.60) which again confirms the involvement of supportive factors by *Acanthamoeba* that enhances the growth of *A. butzleri* many times.

Various studies showing mutual impact of co-culture on both *Acanthamoeba* and bacteria have been carried out. Steinert *et al*., (1998) performed direct co-culture experiments with *A. polyphaga* and *M. avium, L. pneumophila* and *E. coli.* For *M. avium* the results showed an increase from an initial number of 1.5×10^3 CFU for *M. avium* to 3.3×10^3 and 4.3×10^6 CFU after 7 and 14 days, respectively. In case of *L. pneumophila* the number of bacteria increased from 5×10^2 CFU to 3×10^5 and 2×10^7 CFU after 2 and 14 days respectively. For *E. coli* co-culture, the number of bacteria increased from 866 CFU to 1.6×10^6 CFU in just 1 day. The results of parachamber experiments showed similar growth potentials for both *M. avium* and *E. coli* as observed in the direct co-culture experiment. However, *L. pneumophila* failed to demonstrate any increase in number in parachamber experiment. Moreover, the encystation of *Acanthamoeba* also increased as a result of low nutrient content.

In other studies a persistent relationship between *Acanthamoeba* and *T. equigenitalis*/*T. asinigenitalis* has been observed. Allombert *et al*., (2014) studied the effect of co-culturing of *Acanthamoeba* and *T. equigenitalis* or *T. asinigenitalis,* while *E. coli* was used as amoeba-sensitive control bacterium and *L. pneumophila* was used as amoeba-resistant control bacterium by culturing these bacteria separately with *A. castellanii* at an MOI=50 for upto seven days. For taylorellae, the concentrations of extracellular decreased about one fold while for intracellular bacteria these remained constant over the study period. In case of *L. pneumophila* both the extracellular as well as intracellular concentrations rose to the maximum by day-2 followed by a decline until day-7 due to nutrient depletion. In case of *E. coli*, for both extracellular and intracellular bacteria, their number declined constantly until the end of experiment. These results showed a persistent relationship between *Acanthamoeba* and *T. equigenitalis*/*T. asinigenitalis.*

A negative impact of *Acanthamoeba* on bacteria was demonstrated by Iqbal *et al*., (2014). They found that the conditioned medium from *A. castellanii* strongly

exhibited bactericidal properties against methicillin-resistant *Staphylococcus aureus* (MRSA) but limited activity against *Acinetobacter* sp., *P. aeruginosa* and vancomycin-resistant *Enterococcus faecalis* (VRE).

Overall, the results of current study indicate that in the co-cultures of *Acanthamoeba* and *A. butzleri*, the latter are the beneficiaries while the former are negatively impacted. This was in contrary to the observations made for the co-cultures of *A. castellanii* and *E. coli*, *Micrococcus luteus*, *P. aeruginosa*, *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus* and *Enterobacter cloacae.* The presence of bacteria in cultures caused proliferation of *Acanthamoeba* and delayed the onset of encystment in all the cases (de Moraes and Alfieri, 2008).

In brief, the extracellular growth of *A. butzleri* is supported very well by *Acanthamoeba* as it has been observed for *Vibrio parahaemolyticus* (Laskowski-Arce and Orth, 2008). Taken together the results of co-culture experiments of *Acanthamoeba* and *A. butzleri* and the intracellular survival studies (section 3.3.4.) clearly indicate that during the interaction of *A. butzleri* with *Acanthamoeba*, *A. butzleri* enjoys support not only for extracellular survival but also for intracellular survival and protection. This is a matter of concern for an emerging human pathogen like *A. butzleri*.

3.4.6. Effect of *A. butzleri* conditioned media (AbCM) on excystment and encystment of *Acanthamoeba*

The possibility of any substances or factors released in the media (conditioned media) by *A. butzleri* while their culture on the excystment of *Acanthamoeba* was assessed (Figure 3.61 and 3.62). AX2+, VD, (AX2+)+VD and (AX2+)+AbCM (or sup VD-ED) were used a controls. AbCM did not seem to have a positive effect on early and rapid excystment (rather somewhat inhibitory effect) as better and early excystment was observed in case of AX2+ followed by (AX2+)+VD. AbCM not only had least effect on excystment but also the encystment following excystment was rapid indicating an inhibitory effect. Overall, the total cells number (trophozoites and cysts) in AX2+ have a growing trend by the fourth week. While AbCM shows a

declining trend which is slightly lower than the declining trend in VD media. These results were consistent with those of Iqbal *et al*., (2014) who tested the effect of the conditioned media of various bacteria. The conditioned medium of *E. coli* and *Enterobacter* sp. was found to exhibit strong anti-acanthamoebic effects in a concentration-dependent manner.

Based on the findings of current study, it can be concluded that *A. butzleri* do not release any factors in the growth media that can be supportive for improved excystment of *Acanthamoeba* cysts or support their growth after excystment. These findings also supported the findings of co-culture discussed above indicating a negative impact of *A. butzleri* towards *Acanthamoeba*.

3.5. Conclusion

A number of important observations have been made in this study which, taken together, suggest a potential role of *Acanthamoeba* in acting as an environmental reservoir for the emerging human pathogen-*A. butzleri*. Bacterial chemotactic, attachment and internalization findings all suggest *A. butzleri* to act as an easy and most likely a preferred food for *Acanthamoeba*. *A. butzleri* were found to be easily located by *Acanthamoeba* because of the strong chemotactic attraction by amoebae probably because of some factors released by bacteria. *A. butzleri* have strong attachment with the amoeba cells resulting in "capping" of bacteria on the cell surface along with quick internalization although phagocytosis of *A. butzleri* appears to be a complex process that requires involvement of sugar receptors on the surface of *Acanthamoeba*, actin polymerization, PI3-kinases, PTPs and vATPases. The surviving success of *A. butzleri* in phagosome appears to lie in defying of killing processes such as inhibiting lysosomal enzymes, preventing lysosome-phagosome fusion, avoiding phagosome acidification and impacting on the intracellular transport system. *A. butzleri* can exploit amoebae as environmental reservoir not only for survival but also to improve their pathogenic potential as manifested by their ability for limited proliferation/lysis and long term intracellular survival for upto two weeks normally or even upto 42 days as a results of enhanced pathogenic potential conferred by the repeated passages through amoeba cells. This strengthens the previously acknowledged role of amoebae in selection of pathogenic traits of bacteria. *A. butzleri* under altered environmental conditions (presence of nicotinic acid) can change their pathogenic traits towards *Acanthamoeba* and better exploit amoebae as biological reservoirs with better survival. *Acanthamoeba* also support extracellular survival (but not excystment) of *A. butzleri* by releasing growth supporting factors although they are not paid back by *A. butzleri* in the same way. Based upon the interaction studies of *A. butzleri* and *Acanthamoeba*, it is clear that *Acanthamoeba* facilitate *A. butzleri* in all the possible ways. Therefore, *Acanthamoeba* are potential environmental reservoirs and breeding sites for the emerging human pathogens-*A. butzleri*. It is quite possible that *Acanthamoeba* might have played their role in the emergence of virulence traits *A. butzleri* who gained attention as a result of a number of cases and outbreaks of this

pathogen, previously known to be non-pathogenic environmental bacterium. This is concerning because ignoring the role of *Acanthamoeba* as the reservoirs and modulators of pathogenicity trait, can sustain the risk of *A. butzleri* outbreaks. Therefore, the potential role of *Acanthamoeba* in acting as environmental reservoirs for *A. butzleri* should be considered while laying out any control and treatment strategies.

3.6. Further investigations

Although the current study provides a comprehensive view of the impact of *Acanthamoeba* on bacteria with focus on *A. butzleri*, there are still many areas that can be explored further. A detailed study of comparison of *A. butzleri* genes putatively involved in the survival of these bacteria in *Acanthamoeba* and human infection (using various human intestinal cell lines) can further highlight the level of similarity of intracellular survival mechanisms involved in both these types of cells that can further signify the pre-adaptive role of amoebae in selection of virulence traits in *A. butzleri*. More infections of *A. butzleri* using various environmental isolates of *Acanthamoeba* and even other protozoa can broaden the understanding of the role of these free living protozoa in serving as environmental reservoirs for *A. butzleri* and emergence of virulence traits of these bacterial pathogen.

Chapter 4

Impact of *Acanthamoeba* on bacteria-II: Interaction of *Acanthamoeba* with *Rhodococcus equi*

Abstract

This chapter deals with investigating the influence of *Acanthamoeba* on bacteria taking second of the two examples of emerging human bacterial pathogens-Rhodococcus equi. *R. equi*, the Gram+ve coccobacilli, are emerging human pathogens and cause respiratory infections in immunocompromised individuals. The pathogenic potential is associated with the possession of virulence plasmids having pathogenicity island (PI) with virulence associated protein (*vap*) genes; the most important of these is *vapA*. PI is evolutionarily significant as it is believed to have been acquired through horizontal gene transfer (HGT). This gives rise to the hypothesis that "ability to survive in amebae may be pre-adaptive for *R. equi* existence as pathogens in macrophages and thus horizontal acquisition of PI by *R. equi* ancestor (an original commensal) made it pathogenic by conferring the ability for intramacrophagal survival". To test this hypothesis, the intracellular survival pattern of *R. equi* was first studied in macrophages and compared with that in *Acanthamoeba*. Procedures were first optimized in murine macrophages (J774A.1 and RAW264.7) primarily using wildtype (WT) plasmid-bearing (103^{+}) and plasmid-cured (103^{-}) strains which indicated a proliferation trend mainly from t=24h to t=48h. Infection method was then validated using other WT and mutant strains. Before applying the method to study survival of *R. equi* in *Acanthamoeba* (MN-7), some modifications were made to compensate for the detached cells at 37°C. *Acanthamoeba* MN-7 were then infected with *R. equi* WT strains 103^{+} and 103^{-}. Compared to macrophages, the rate of internalization was low in *Acanthamoeba*. Infections performed at 37°C (virulence genes activated) vs 22°C (virulence genes inactivated) showed that *R. equi* had potential for limited proliferation and survival in macrophages in a temperature and plasmid dependent manner (no survival at 22°C and no survival in plasmid cured 103^{-}). These findings were also confirmed with experiments at 34°C (minimum temperature with notable expression of virulence genes as determined by gene expression experiments). *In vivo* expression of virulence genes (*vapA*, *vapC* and *vapF*) confirmed a comparable activation of these genes in macrophages as well as in amoeba cells. These results indicate that although *R. equi* show limited survival potential in amoeba cells as compared to macrophages yet they share the basic strategies which seems to support the hypothesis. Furthermore, ability of amoebae to support the growth of plasmid-bearing *R. equi* indicates their role in acting as environmental reservoirs which can pose a threat for human and animal health.

4.1. Introduction

Rhodococcus equi, the multihost pathogen, once thought to only infect equines, has recently emerged as life-threatening opportunistic pathogen in humans with compromised immunity (Cohen *et al*., 2015; Puthucheary *et al*., 2006). These Gram+ve actinomycete rhodococci are of great interest because of their diverse activities, being capable of metabolizing a wide range of environmental pollutants including some toxic chemicals (like nitroaromatic compounds and polychlorophenols) and synthesizing commercially important products (as acrylamide) (Bell *et al*., 1998). Due to their mycolic acid containing cell wall, rhodococci are grouped into mycolata, which also includes *Mycobacterium*, *Nocardia* and *Corynebacterium* (Gurtler *et al*., 2004).

R. equi are present in the soil as well as faeces of grazing herbivores and use manure for growth. The transmission takes place through inhalation of bacteria either in contaminated dust or directly from an infected animal. *R. equi* can replicate in intestines resulting in mesenteric lymphadenitis. Ingestion of bacteria is also considered a route of infection in which case the ingested bacteria passage through the alimentary canal. The virulent bacteria are disseminated from the infected foals through faeces.

The important pathogenic aspect of *R. equi* is their ability to parasitize the macrophages, where they live in membrane bound vacuole called *R. equi* containing vacuole (RCV), and eventually destroy the macrophages. Virulence is related to the presence of plasmids (80-90 kb) carrying genes for virulence-associated proteins (Vaps), the most important of which in foals is VapA (Barton and Hughes, 1984; Takai *et al*., 1999; Takai *et al*., 2000; Jain *et al*., 2003). *R. equi* are a serious threat to the equine breeding industry due to their high incidence, abundance in the farm environments and unavailability of an effective vaccine (Giguere *et al*., 2011; Muscatello *et al*., 2007).

4.1.1. *Rhodococcus equi*

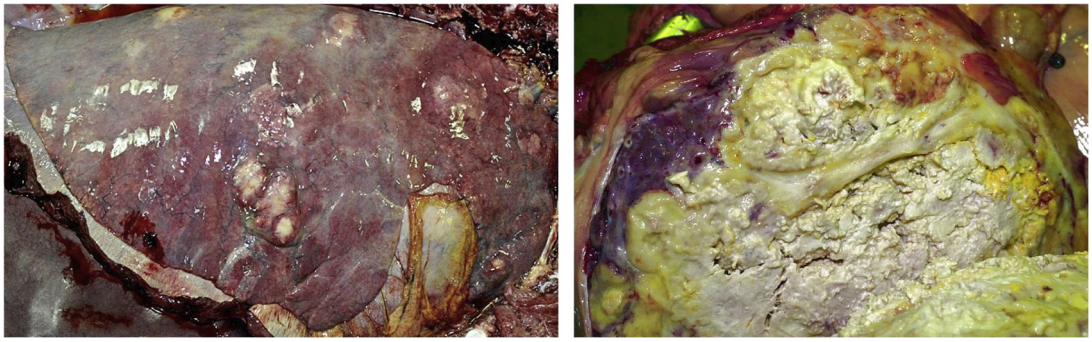
R. equi may appear differently depending upon the growth conditions and cycle being coccoid (on solid media and in purulent lesions) or filamentous branched rods in broth media. (Prescott, 1991). *R. equi* are non-flagellated (Prescott, 1991), but some strains can have appendages or pili (Nordmann *et al*., 1994). The optimal temperature for growth of *R. equi* ranges between 30-37°C (Prescott, 1991) but they can grow between 10-40°C (Walsh *et al*., 1993). *R. equi* can use carbon from different sources such as organic acids like proprionate or acetate, which are abundantly present in manure of herbivores (Prescott, 1987; Prescott, 1991). *R. equi* can also use lipids as a source of carbon which is reflected by the presence of many chromosomal genes involved in lipid metbolism like mycobacteria but interestingly no genes for sugar transport. *R. equi* are surrounded by thick polysaccharide capsule (Prescott, 1991) and they can survive some extreme environmental conditions like oxidative stress and low pH (Benoit *et al*., 2002; Benoit *et al*., 2000).

4.1.2. *R. equi* infections

R. equi cause "rattles", a lung disease with a high mortality in foals, which is the most common cause of lung abscesses and mortality (Hillidge, 1987; Lavoie *et al*., 1994; Muscatello *et al*., 2007). *R. equi* infection appears as pneumonia in the form of chronic suppurative bronchopneumonia resulting in widespread abscesses and lymphadenitis (Figure 4.1-a). It affects upto 15% foals with 6% case fatality rate Muscatello *et al*. (2007). Infected foals have fever and respiratory distress while lung abscesses filled with pus are usually seen (Lavoie *et al*., 1994) and if left untreated may cause asphyxiation and death. In addition to respiratory infection, ingestion or introduction of the organism into cuts can cause intestinal or wound site ulcers (Bell *et al*., 1998). The disease can also be manifested in less common acute form in which the foals may die in a day or within few days showing only respiratory distress (Martens *et al*., 1982; Prescott, 1991).

The *R. equi* infection may not just be limited to lungs and can affect various other organs resulting in ulcerative enteritis, typhlitis (over Peyer's patches), inflammation of mesenteric/colonic lymph nodes, osteomyelitis, arthritis and abscesses of liver and

kidneys (Johnson *et al*., 1983; Paradis, 1997; Zink *et al*., 1986). In some cases, the only abdominal lesion can be a mesenteric node abscess (Figure 4.1-b).



(a) (b)

Figure 4.1: The lung from a foal infected by *R. equi* with severe purulent pneumonia, showing numerous abscesses (a). An abdominal abscess (cross section) recovered from a foal with *R. equi* infection (b). Taken from Vazquez-Boland *et al*. (2013).

Adult horses seem protected and are rarely infected (Hondalus, 1997), however, *R. equi* can be detected in many adult horses without clinical signs. *R. equi* are also responsible for equine abortion (Nakamura *et al*., 2007; Patterson-Kane *et al*., 2002; Szeredi *et al*., 2006). *R. equi* infect immuno-compromised humans with high mortality and the most commonly affected organ is lung (Bell *et al*., 1998; Hsueh *et al*., 1998). *R. equi* can also cause respiratory diseases in a wide range of other animals including pigs, cattle, goats, sheep, llama, cats and dogs (Muscatello *et al*., 2007; Prescott, 1991).

4.1.3. Virulence of *R. equi*

It is known that the virulence of *R. equi* is directly related to the presence of the virulence-associated plasmids (Takai *et al*., 1992; Takai *et al*., 1991; Takai *et al*., 1993; Tkachuk-Saad and Prescott, 1991). The pathogenicity island (PI) of *R. equi* contains many genes but the most important is the virulence associated protein (*vap*) family consisting of seven genes including *vapA*, *vapC* and *vapF* (Polidori and Haas, 2006; Takai *et al*., 2000) together with two pseudo-*vap* genes (Russell *et al*., 2004). The *vapA* is the most important, and encodes a 15-17 kDa virulence-associated protein A (VapA) (Jain *et al*., 2003) which is required for survival and growth in macrophages (Giguere *et al*., 1999; Jain *et al*., 2003). Several copies of plasmids may

be present but can be lost at 38°C in broth cultures if repeatedly passaged (Chirino-Trejo and JF, 1987; Takai *et al*., 1991). The expression of *vapA* and other genes of the PI is induced in conditions normally encountered in phagosomes (i.e. low pH, oxidative stress and low levels of divalent cations such as $Fe^{2+,}$ Ca^{2+} and Mg^{2+}) and a high temperature (Benoit *et al*., 2001; Benoit *et al*., 2002; Jordan *et al*., 2003; Ren and Prescott, 2003; Takai *et al*., 1996; Takai *et al*., 1992).

4.1.4. Niche plasticity

In *R. equi* the genome is rich in G+C content (68.8%) and it is smaller than that of most environmental species, which is the results of niche-adaptation (intracellular mode of life) (Letek *et al*., 2010). The chromosome is 5043 kb (circular) and lacks DNA mobility genes. The virulence plasmid is designated as pVAP and is the main determinant to virulence (Giguere *et al*., 1999; Hondalus and Mosser, 1994; Takai *et al*., 1991). The virulence plasmids (pVAP) are 80-100 kb and harbour the PI which plays a central role in pathogenicity (survival in macrophages) and is believed to be acquired through horizontal gene transfer (HGT) (Letek *et al*., 2008). The pVAP plasmid belongs to the CURV family (name derived because of the properties of **c**onjugation, **u**nknown-function, **r**eplication and **v**ariable region modules) and comprises, among others, a niche-adaptive variable region (VR) which is horizontally acquired and plays a vital role in niche adaptation. For example, while in *R. equi* this VR corresponds to the PI, in biodegrading species it is responsible to carrying out catabolic functions (Letek *et al*., 2010).

Overall, the genome of *R. equi* exhibits various features for niche adaptation, apart from virulence genes, which distinguish it from most environmental species. These include the absence of catabolic genes found in environmental species, and the presence of genes encoding for a number of proteins (secretary as well as surface displayed) and other regulators. These characteristics enable *R. equi* to lead a saprophytic as well as pathogenic mode of life. Furthermore, although *R. equi* is aerobic but it can also survive under anaerobic environment by using utilizing nitrate/nitrite (Letek *et al*., 2010). These robust features make this pathogen significant in human and animal health perspectives.

4.1.5. Interaction between *R. equi* and *Acanthamoeba*

As discussed in detail in Chapter 1 (section 1.1.9.2.3.) the interaction between *Acanthamoeba* and bacteria is of great importance owing to the role of amoebae in acting as an environmental reservoirs whereby they not only provide protection to the bacteria but also bacteria are under pressure of developing strategies to improve their pathogenic potentials for better survival. This is of great significance in the case of emerging human pathogens like *R. equi* which infect macrophages. There are remarkable similarities between amoebae and macrophages; both ingest microorganisms by phagocytosis and both share basic strategies. Amoebae are thus considered primitive macrophages. *Acanthamoeba* offer a model to study the interaction of intracellular microorganisms with phagocytic cells in the environment (Vieira *et al*., 2015).

R. equi have their virulence dependent upon the PI on the plasmid. It is interesting to note that the sequence of the PI is different from the rest of the plasmid, indicating the acquisition of this important part possibly through horizontal gene transfer (Letek *et al*., 2010; Vazquez-Boland *et al*., 2013) from another organism which probably had the potential to survive the hostile cellular environment of protists (amoeba) thereby acquiring the ability to survive the primitive macrophages (amoebae).

It is further hypothesized that the strategies learnt for intracellular survival in amoebae were later used by *R. equi*, through co-option of core bacterial structures and functions, to infect animals by managing to survive in their macrophages (Figure 4.2).

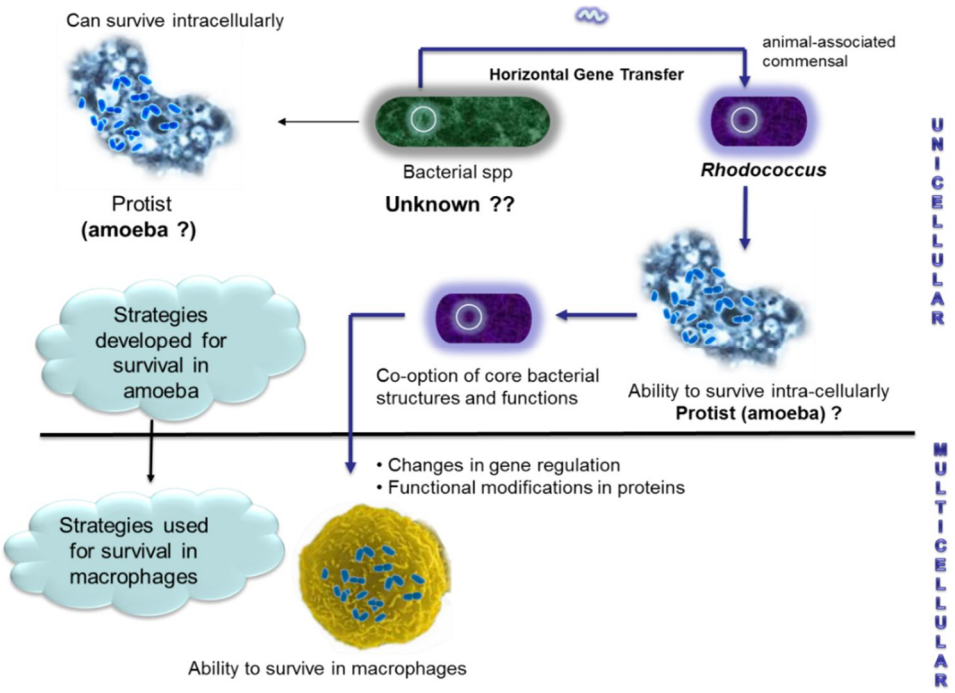


Figure 4.2: A diagrammatic representation of the possible picture of the significance of interaction of *R. equi* with amoebae. *R. equi* is believed to the just an animal associated commensal who acquired the virulence traits through horizontal gene transfer from another pathogen that could survive the hostile cellular environment of some protist (amoeba) enabling it to survive inside the amoebae. Through the evolutionary process by the co-option of core bacterial structures and functions along with the changes in gene regulation and functional modifications in proteins, the strategies developed to survive in amoebae was later employed to infect the macrophages of animals and humans.

If this is true then it means that the possible evolutionary role of amoebae in adaptation of *R. equi* to the intracellular environment which enabled them to infect macrophages and emerge as a potential human pathogen may be traced back by using the amoebae as the "original" host model. Moreover, in amoebae *R. equi* should demonstrate somewhat comparable intracellular survival machinery and strategies to as used for macrophages. Therefore, *Acanthamoeba* can provide the missing link between ecology and pathology of *R. equi* just like it has been observed with other bacteria like *Legionella pneumophila* and *Mycobacterium* sp. (Chapter 3, section 3.1.1.). This gives rise to the following hypothesis to be tested:

"Ability to survive in amebae may be pre-adaptive for the existence of *R. equi* as pathogens in macrophages and thus horizontal acquisition of PI by *R. equi* ancestor (an original commensal) made it pathogenic by conferring the ability for intramacrophagal survival."

To test this hypothesis, it is required to carry out infections of *Acanthamoeba* with *R. equi* to understand whether the virulence plasmid, which is believed to be horizontally transferred, and plays vital role in survival of *R. equi* in *Acanthamoeba* also plays role in protecting these bacteria in *Acanthamoeba*. However, before that, it was important to carry out infections in macrophages using an optimized method to understand, for comparison under the laboratory conditions, the survival pattern and key determinants involved.

4.1.6. Objectives of the study

The study of the interaction between *R. equi* and *Acanthamoeba* can have dual impact. On one hand the evolutionary significance of amoebae and HGT in niche adaptation of *R. equi* from a saprophytic mode of life to acquisition of characteristics of a pathogen can be studied. While on the other hand the potential of amoeba as an environmental reservoir for *R. equi* and the potential for modulation of the pathogenic traits of this emerging human pathogen can be reviewed. The key objectives of this study can be listed as follows:

- To optimize and validate an infection assay for the macrophages (site of infection of *R. equi*) and *R. equi* WT and mutant strains. Also to find out the best conditions for monitoring intracellular survival of *R. equi* in *Acanthamoeba*.
- To work out the precise *in vitro* expression of virulence genes as a function of changing temperature to find the least temperature, with adequate virulence genes expression, to be used for *Acanthamoeba* infection assays.
- To study the role of virulence plasmid and temperature in the survival of *R. equi* in *Acanthamoeba*.
- To compare the *in vivo* upregulation of important virulence genes of *R. equi* after infection in macrophages and *Acanthamoeba*.
- To assess the significance of *Acanthamoeba* in acting as environmental reservoirs for *R. equi*.

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4.2. Materials and methods

4.2.1. Bacterial strains and culture conditions

The wild type and mutant strains of *R. equi* used are summarized in Table 4.1 were kindly provided by Prof Jose V Boland, Ashworth Laboratories, University of Edinburgh.

Table 4.1: List of *R. equi* wildtype and mutant strains used for the current study.

***R. equi* strain/ mutant**	**Description**
103^+	Equine isolate; wild type strain (with an 85 kb virulence plasmid harbouring *vapA*)
103^-	Plasmid-cured isogenic derivative strain of 103^+
RE1	Equine isolate; wild type strain also harbouring *vapA*
PAM 1571	Wildtype, bovine isolate harbouring pVAPN plasmid
PAM 1593	Wildtype, pigs isolate harbouring pVAPB plasmid.
arsB	Mutant strain; derived from 103^+ by disruption of *arsB* gene with apramycin resistance gene
supAB	Mutant strain; derived from 103^+ by deletion of neighbouring *supA and supB* genes together
mce1	Mutant strain; ; derived from 103^+ by in-frame deletion of *mce1* gene
choE	Mutant strain; derived from 103^+ by in-frame deletion of *choE* gene
chori	Mutant strain; derived from 103^+ by in-frame deletion of REQ23860 gene.
ureA	Mutant strain; derived from 103^+ by in-frame deletion of urease encoding REQ45360-410

All the bacterial strains were cultured in brain-heart infusion (BHI) at 37°C (Ladron *et al*., 2003). However, for gene expression experiments, *R. equi* were cultured in Luria-Bertani (LB) medium. Before using a stock of bacteria for the infection assays these were first tested for their sterility (by streaking on BHI agar plates), growth capacity (in BHI over a period of 24 h) and finally checking for the plasmid integrity (section 4.2.4.) for which purpose a PCR based identification was performed targeting two plasmid genes *vapA* and *traA* while the chromosomal gene *choE* as control which is highly conserved chromosomal locus in *R. equi* encoding for cholesterol oxidase (Ladron *et al*., 2003).

All the bacterial strains were cryopreserved using 20% glycerol. These were grown in BHI at 30ºC to and OD_{600}=1.0 and were washed twice with PBS and reconstituted in 20% buffer glycerol (20% glycerol in PBS). Small aliquots of these were then made and preserved at -80ºC. For revival, a cryopreserved tube was taken out and defrosted. The bacteria were then washed twice with PBS and reconstituted in BHI broth. These bacteria were then streaked onto the surface of the BHI agar plate and incubated at 30ºC overnight. A single colony from the revived bacteria was then picked up and transferred to 10 mL BHI broth in Falcon tube. The bacteria were then cultured in a shaking incubator at 30ºC overnight. These bacteria were then streaked on BHI agar plates and incubated again at 30ºC overnight with growth confirmed by the appearance of bacterial colonies. The plates were sealed and stored in the refrigerator for immediate experiments. For the experiment purpose, a single colony was picked and cultured in BHI broth at 37ºC by growing to OD_{600}=1.0.

4.2.2. Macrophage cell lines, growth conditions, preservation and revival

Murine macrophage cell lines J774A.1 (ATCC, TIB-67) and RAW 264.7 (ATCC, TIB-71) were used. The former was provided by Prof Jose V Boland, Ashworth Laboratories, University of Edinburgh while the latter was obtained from Roslin Institute, University of Edinburgh. The cells were cultured in an incubator at 37ºC and 5% CO_2. The base medium for both the cell lines was DMEM (Invitrogen) supplemented with 10% foetal bovine serum (Lonza) and 2 mM glutamine (Gibco).

For preservation of macrophages, complete growth medium supplemented with 5% DMSO was used. The cells were cryopreserved at -80ºC. For revival of cells, a cryopreserved tube was taken out and immediately transferred to a 37ºC water bath. In a sterilized safety cabinet, the cells were taken out of the tube and transferred to 15 mL Falcon tube with 10 mL of growth medium. The cells were centrifuged at 1000 rpm for 10 min to pellet the cells. The supernatant was discarded and the cells were reconstituted with fresh medium 6 mL. This cellular suspension was then transferred to 25 cm^2 cell culture flask and incubated at 37ºC. The cells were monitored after 24h and fresh medium was replaced with old medium. Near confluence, the cells were

scrapped off the surface and transferred to 75 cm^2 cell culture flask while a small aliquots was taken out to check for the *Mycoplasma* contamination (Letek *et al*., 2010).

4.2.3. *Acanthamoeba* strains and culture conditions

Acanthamoeba strains used for this study included *A. castellanii* MN-7 (human mesenteric node isolate) and was kindly provided by Dr Sutherland K Maciver, School of Biomedical Sciences, University of Edinburgh. ***AX2+ media*** for *Acanthamoeba* culture was prepared as mentioned in Chapter 2 (section 2.2.1.).

4.2.4. Plasmid integrity of *R. equi*

The plasmid integrity of *R. equi* is crucial for its virulence and was determined by a PCR based identification targeting two plasmid genes *vapA* and *traA* while a chromosomal gene *choE* which encodes for cholesterol oxidase (Ladron *et al*., 2003; Ocampo-Sosa *et al*., 2007; Takai *et al*., 1998) was also included as control (Table 4.2).

Table 4.2: The primers used for the amplification of *R. equi* plasmid genes (*vapA*, *traA*) and chromosomal gene (*choE*) to confirm the presence of plasmid.

Primer pair	Sequence (5´-3´)	Annealing temp (°C)	Extension time (sec)	Product size bp)	Reference
*vap*_IP1 *vap*_IP2	gactcttcacaagacggt taggcgttgtgccagcta	56	60	512	Takai *et al*., 1998
traA-F1 *traA*-R1	agagttcatgcgtgacaacg gtccacaggtcaccgttctt	56	90	959	Ocampo-Sosa *et al*., 2007
*choE*_COX-F *choE*_COX-R	gtcaacaacatcgaccaggcg cgagccgtccacgacgtacag	56	90	959	Ladron *et al*., 2003

The mastermix for PCR amplification consisted of 2.5 µL of DNA template, 0.5 µL of 1U/µL *Taq* DNA polymerase (Biotools, Spain), 0.5 µL of dNTPs (Biotools, Spain), 2.5 µL of reaction buffer 10× (Biotools, Spain), 1.25 µL each of the set of primers (Sigma, UK) at a working concentration of 10 µM, and 16.5 µL of PCR grade water (Sigma, UK). After initial denaturation at 95°C for 5 min the DNA was subjected to 29 amplification cycles under the following conditions: denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 60-90 sec (Table 4.2). The reactions were terminated after the final cycle, by an extra run at 72°C for 10 min, and then the mixtures were kept at 4°C until analyzed.

4.2.5. Test for *Mycoplasma* contamination in macrophage cell lines

A PCR based assay was performed routinely for detecting *Mycoplasma* contamination (Schmitt and Pawlita, 2009). DNA extraction was achieved by using GenElute™ Blood Genomic DNA Kit (Sigma) as per manufacturer's instructions. The amplification of DNA for detection of *Mycoplasma* was performed using the Venor®GeM *Mycoplasma* Kit for conventional PCR (Minerva Biolabs GmbH, Berlin) except the *Taq* DNA polymerase (Sigma, UK) as per manufacturer instructions.

4.2.6. Nitric oxide (NO) determination

The concentration of NO was measured by Griess method as described by Guevara *et al*., (1998) with some modifications. This involved addition of equal volumes (50 µL) of sulfanilamide (1%) to cell culture supernatant followed by incubation of 5 min at RT under reduced light. To this reaction mixture 50 µL of NED solution (0.1%) was added and incubated under the same conditions as before. Samples were analyzed at 540 nm in a microplate spectrophotometer and the nitrite concentration was calculated with the help of standard curve for sodium nitrite (0-100 mM).

4.2.7. Assay for infection of macrophages with *R. equi*

The layout of this method was designed in our laboratory using the minimum variables. For this purpose murine macrophages (J774.A1) were cultured in 24-well plate (2×10^5 cells/mL/well) in the growth medium (DMEM with 2 mM glutamine and 10% foetal bovine serum) and incubated at 37ºC, 5% CO_2 overnight. The number of viable cells per well was counted and resuspended in the appropriate amount of washed bacteria in DMEM to have the required MOI of 10 bacteria per cell. The medium already present in the 24-well plate was removed and 1 mL of reconstituted bacteria was added. The plate was then centrifuged for 3 min at 900 rpm and incubated at 37ºC in 5% CO_2 for 45 min.

After that the medium was removed, cells were washed three times with PBS and complete growth medium containing 5µg/mL vancomycin to kill any extracellular bacteria was added. One hour from this point was taken as time zero (t=0) at which time macrophage monolayers were washed twice with PBS. For lysing the

macrophages to release the bacteria, 200 µL of 0.1% triton was added to each well and left for 3 min followed by addition of 800 µL of PBS and thorough mixing. This suspension was transferred into an Eppendorf tube and bacterial colonies were titred by serial dilutions on BHI agar plates to find the colony forming units (CFUs) of *R. equi* per well or mL. The plates were incubated for 48 h and the medium was changed after 24 h with fresh growth medium containing 5 µg/mL vancomycin HCl. A total of three readings were made at time 0, 24 and 48h.

4.2.8. Assay for infection of *Acanthamoeba* with *R. equi*

Because of comparatively slightly reduced attachment of *Acanthamoeba* MN-7 cells with the 24-well plate at 37°C than at 22°C, some procedural modifications had to be made in the infection assay for *Acanthamoeba* so that at each time point the number of bacteria recovered also takes into account the number of cells in the respective well.

4.2.8.1. Procedural changes in infection assay for *Acanthamoeba* and *R. equi*

R. equi have temperature dependant activation of plasmid genes so they need exposure to higher temperatures to activate fully their virulence genes. However, on the other hand, *Acanthamoeba* usually have tendency to grow well at lower temperatures. At higher temperature, amoebae seemed to detach from the surface of the plate. Due to this behaviour, some changes were made in the protocol for studying interaction of *Acanthamoeba* with *R. equi* (Figure 4.3).

To start with the infection assay, *Acanthamoeba* (MN-7) cells were seeded into 24-well plate at 4×10^5/mL/well and left overnight to allow the cells to adhere to the surface. Before infection, the supernatant was removed and the monolayers washed once with NS. Washed bacteria were then added to each well at MOI=100. The plate was centrifuged for 3 min at 900 rpm. The plate was then incubated for 1h to allow the amoeba cells to phagocytose bacteria. The bacterial suspension was then removed and the monolayers were washed with NS three times to eliminate majority of the bacteria. Fresh medium with vancomycin at 5 µg/mL was added to kill the remaining extracellular bacteria for 1h. The medium was then removed and the cells were washed with NS twice. At this step, instead of lysing the cells to recover bacteria, either 500

µL (or 250 µL) of PBS was added to each well and the cells were scrapped off, out of which 30 µL was used for cell counting while the remaining 470 µL (or 220 µL) of cell suspension was centrifuged to pellet the cells. The supernatant was removed and the cells were lysed with 100 µL of 0.1% triton X-100 for 5 min followed by dilution with 400 µL of PBS. This was then serially diluted 10-fold and plated on BHI agar plates for colony counts. This was then followed by processing of the data.

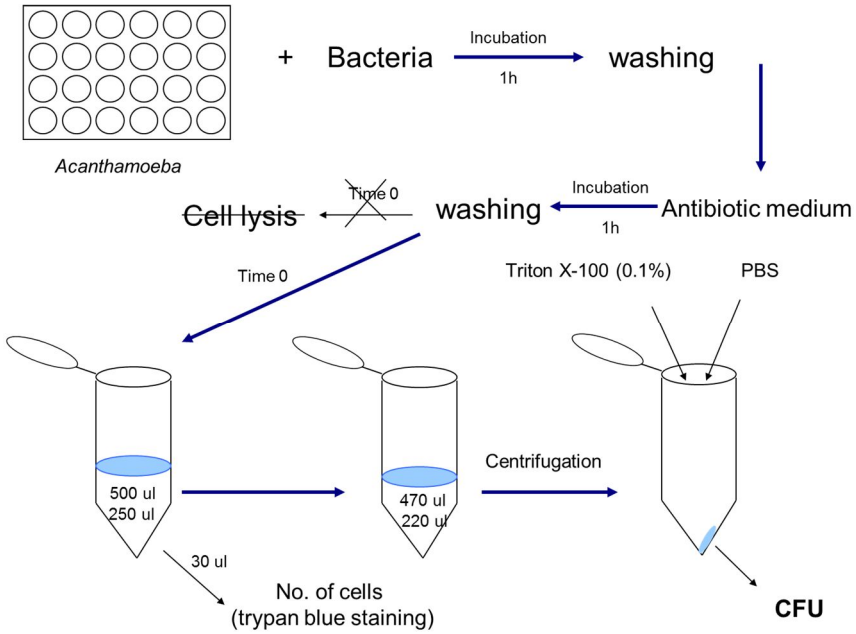


Figure 4.3: Procedural modifications in the infection protocol for *Acanthamoeba* and *R. equi* for more reliable intracellular bacterial survival monitoring. Unlike macrophages, *Acanthamoeba* MN-7 cells did not show firm attachment with the 24-well plate surface. The modified method started as for macrophages with certain changes. This involved preparation of monolayers in 24-well plate (4×10^5 amoebae/well/mL) by leaving amoebae in plate overnight. Following washing of monolayers these were infected with washed bacteria at MOI of 100:1. The plate was centrifuged and left for 1h. After discarding the bacterial suspension, each well was washed thrice and fresh AX2+ with vancomycin at 5 µg/mL was added to kill remaining extracellular bacteria. After 1h of incubation the supernatant from each well was removed and the monolayers washed. At this step, instead of lysing the cells, 500 µL (or 250 µL) PBS was added to each well. Amoebal cells were taken out; 30 µL was used for cell count while remaining 470 µL (or 220 µL) was centrifuged and the pellet was lysed by 0.1% triton X-100 for CFU count. CFU count at each time point was then linked to cell count.

To compensate for any cell loss during the procedure and for normalization of the data, at each time point a ratio was obtained by dividing number of bacteria with the number of amoeba cells (Figure 4.4):

$$\text{Normalized value at each time point} = \frac{\text{No. of bacteria/well}}{\text{No. of } \textit{Acanthamoeba} \text{ cells/well}}$$

Therefore, the graph was plotted between time (h) and number of bacteria/number of cells instead of time (h) and CFUs/well for macrophages.

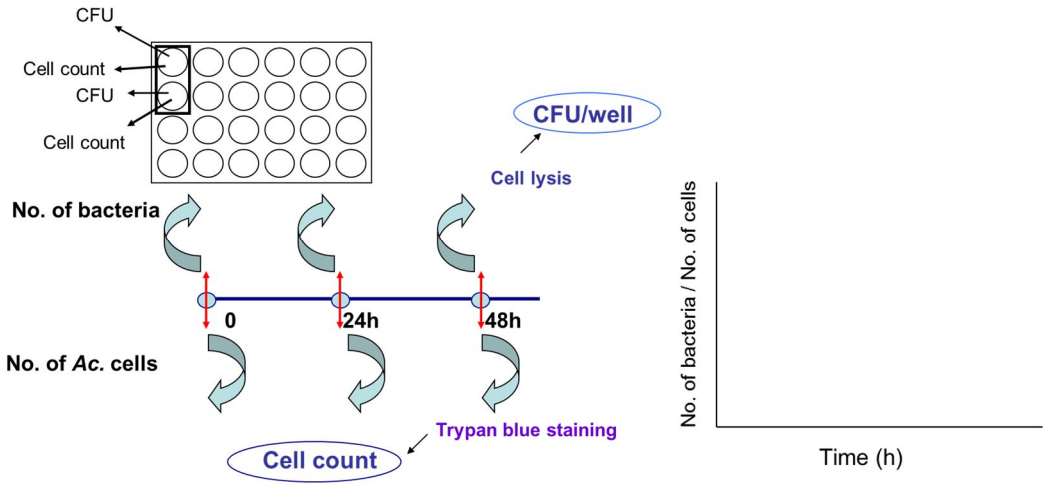


Figure 4.4: Normalization of data for the modified procedure for the infection assays for *Acanthamoeba* and *R. equi* at 37°C. At each time point cell counts were also recorded in addition to CFUs per well. The normalization of data was then carried out by dividing the number of bacteria in each well by the number of amoeba cells in the same well (a). This normalized data was then used to plot graph between Time (h) and number of bacteria / number of amoeba cells (b). *Ac=Acanthamoeba.*

4.2.8.2. Role of plasmid & temperature in survival of *R. equi* in *Acanthamoeba*

The role of plasmid and temperature in survival of *R. equi* in *Acanthamoeba* was measured in this experiment. Whether or not plasmid had a role in the survival of *R. equi* in *Acanthamoeba*, could be assessed by comparing intracellular survival of plasmid-bearing and plasmid-cured strains of *R. equi* in *Acanthamoeba*. Similarly, the role of plasmid activation could be analysed by survival or no survival at the virulence genes activation (37°C) and inactivation (22°C) temperatures.

For this purpose, a comparative experiment at 37°C and 22°C was designed using the plasmid-bearing (103^{+}) and plasmid-cured (103^{-}) strains of *R. equi* (Figure 4.5).

Infection assays were performed as described above (section 4.2.8.1.). For the 22°C experiment, the plate was kept at 22°C through the course of experiment right from the start. However, in case of 37°C experiment, the plate was kept at 22°C until t=0. Thereafter, the plate was moved to 37°C. This was important so that the starting point in both the cases is exactly the same before separating them to different temperatures. The infection experiments were completed until 48h and the results obtained by counting CFUs at each time point were presented in the form of graphs plotting time (h) against number of bacteria/number of cells (y-axis).

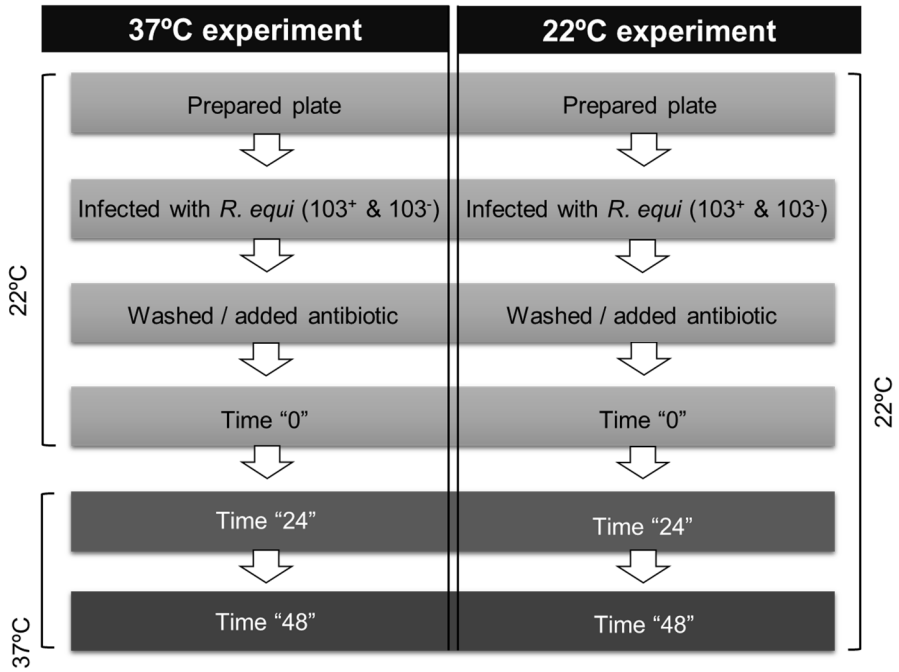


Figure 4.5: Experimental layout to investigate the role of *R. equi* virulence plasmid and temperature in the survival of *R. equi* in *Acanthamoeba* (MN-7). Parallel infections were carried out at 37°C and 22°C using the WT plasmid-bearing (103^+) and plasmid-cured (103^-) strains of *R. equi*. If there was role of plasmid in the survival of *R. equi* in *Acanthamoeba*, this could be assessed by comparing results for plasmid-bearing and plasmid-cured strains while the role of plasmid activation could be analysed by survival or no survival at the virulence genes activation (37°C) and inactivation (22°C) temperatures. Plates were seeded with 4×10^5 amoebae/mL/well in 24-well plates and allowed to settle down overnight. Infections with the bacterial strains were carried out at MOI=100:1. After 1h incubation at 22°C, bacteria were removed and monolayers washed with NS followed by addition of AX2+ medium with 5 µg/mL vancomycin and 1h incubation at the same temperature. One hour from this point was t=0 when the monolayers were washed twice with NS and the bacteria were recovered by lysing cells with 0.1% triton X-100 and plating the 1:5 diluted lysate onto the agar plates to count CFUs.

4.2.9. Gene expression of *R. equi*

The various procedures used for the determination of *in vitro* and *in vivo* gene expression profile of *R. equi* were adopted from Letek *et al*. (2010) using the RT-PCR method of relative gene expression with SYBR Green. The selection of genes to be used was made on the basis of microarray expression analysis of plasmid genes (Letek *et al*., 2010). The most expressed genes were used for comparative expression at various temperatures while the non-PAI genes were used as controls as they are not affected by the temperature. Four plasmid pathogenicity island genes (pVAPA_*vapA*, pVAPA_*vapC*, pVAPA_*vapF*, pVAPA_0560) were included.

4.2.9.1. Bacterial culture and sample preparation

A 50 mL volume of LB medium was inoculated with a single colony of *R. equi* and cultured overnight, with continuous shaking at required temperature, to stationary phase. A small volume of this culture was used to prepare a fresh culture in LB medium in 50 mL LB with start OD_{600} ~ 0.02. Bacteria were harvested to OD_{600}=0.8-0.1 and centrifuged at 4°C at 3220 ×*g* for 5 min. The supernatant was removed and the pellet was resuspended in 1 mL Tri Reagent (Sigma). The suspension was transferred to a 2 mL screw-capped tube containing 500 µL DEPC-treated 0.1 mm zirconia-silica beads (FastPrep-24 lysing matrix) and homogenized in a FastPrep apparatus (MP Bio) by beating for 2×30sec pulses at 6.5 speed setting. The tube was then allowed to cool down at RT for 5 min and stored at -80°C overnight. This was then followed by chloroform-isopropanol extraction.

4.2.9.2. RNA extraction and quality testing

During the extraction procedure, for the DNAase treatment, Turbo DNA-free kit (Ambion) was used while for purification RNeasy kit (Qiagen) was used. The frozen samples were thawed on ice, centrifuged for 1 min at 16,100 ×*g* (RT) and the supernatant was transferred to new Eppendorf tube. A 200 µL volume of chloroform was added and the tube shook vigorously for 15 sec. The layers were allowed to separate for 15 min at RT. The tubes were then centrifuged at 12,000 ×*g* (4°C) for 15 min. The clear upper aqueous phase (~700 µL) was transferred to a new Eppendorf tube. A 500 µL volume of isopropanol was added, tubes mixed well, allowed to precipitate at RT for 10 min and centrifuged at 12,000×*g* (4°C) for 10 min. The

supernatant was removed, 1.5 mL 75% ethanol added and tubes centrifuged at 7,500 ×*g* (4°C) for 5 min. The supernatant was removed and the tubes were air dried for 10-15 min followed by resuspension with 90 µL H_2O and incubation at 65°C for 5 min. Then 10 µL Ambion Turbo 10× Buffer and 2 µL Turbo DNase were added and tubes incubated at 37°C for 30 min. Qiagen buffer RLT (350 µL) was then added, the tubes mixed thoroughly and 250 µL of absolute ethanol added with thorough mixing.

The samples were then applied to RNeasy column, centrifuged at 8,000 ×*g* for 15 sec followed by addition of 350 µL RW1 reagent to the column, centrifugation at 8,000 ×*g* for 15 sec. Then 10 µL Qiagen DNase I was added to 70 µL Buffer RDD in new Eppendorf and mixed gently. This DNase mix was added to the column, incubated at RT for 15 min and 350 µL RW1 reagent was added to the column. After centrifugation for 15 sec at 8,000 ×*g*, 500 µL RPE reagent was added to the column. Following centrifugation for 15 sec at 8,000 ×*g* 500 µL RPE reagent was added to the column with subsequent centrifugation for 15 sec at 8,000 ×*g*. The flow-through was discard and the columns were centrifuge for further 2 min at 8,000 ×*g*. The columns were transferred to new Eppendorf tubes. A 50 µL volume of RNase-free water was added followed by incubation for 5 min (RT) and centrifugation (8,000 ×*g*, 1 min). The RNA was finally eluted by the addition of 50 µL RNase-free water, incubation for 5 min (RT) and centrifugation (8,000 ×*g*, 1 min).

Quantification of the eluted RNA was made with NanoDrop (Thermo Scientific). If the RNA was very concentrated (>0.8 µg/µL), it was diluted before setting up the reaction. To rule out DNA contamination of RNA, a standard PCR was performed using *vapA* and *choE* primers using RNA as the template. For automated analysis of RNA, QIAxcel Electrophoresis System (Qiagen) was used. RNA samples were treated as per kit instructions and were placed in PCR tubes strip before loading into the machine. Samples were analysed in about 10 min. The 23S:16S ratio was used to find out the relative integrity number (RIN).

4.2.9.3. cDNA synthesis

RT reactions were performed using the *Taq*Man Reverse Transcription Reagents kit (Life Technologies) consisted of the following components:

RT Buffer (10×)	1 µL
$MgCl_2$ (25 mM)	2.2 µL
dNTPs Mixture (2.5 mM)	2.0 µL
RNase Inhibitor (20 U/L)	0.2 µL
MultiScribe Reverse Transcriptase (50 U/µL)	0.25 µL
Reverse Primer (50 µM)	2.5 µL
RNA (0.25 µg)	Variable
RNase-free water	Variable
Total	10 µL

The RT mastermix was prepared by combining all the non-enzymatic components (RT Buffer, $MgCl_2$ and dNTPs Mixture) followed by brief vortexing and addition of the enzymatic components (MultiScribe Reverse Transcriptase and RNase Inhibitor) and mixing of the components. Then the reverse primer was added and 8.15 µL of the mastermix was distributed into each of the PCR tubes. RNA and water were added and the plate was transferred the to the thermal cycler block. The RT reaction was performed with the following protocol:

	Incubation	RT	RT inactivation
Time (min)	10	30	5
Temperature (°C)	25	48	95

After thermal cycling was complete, the samples were stored at -20°C or 4°C depending on the storage time required.

The primers for SYBR green RT-PCR were designed by Primer Express (Table 4.3). Care was taken to have the primers free from problems like primer dimmers, hair-pins, ΔG etc. The ΔG values were specially focused and if found very low the primer was redesigned. Furthermore, the primers were also checked by carrying out a standard PCR using total DNA as the template. For cDNA synthesis random primers were used**.** In addition to these, all the primers were further checked by running them on RT-PCR with all the reagents except cDNA which was replaced by water to rule out any non-specific amplification which might interfere with the results. Primers giving such

amplifications (where non-specific amplification started as early as 30 cycles) were not used and were redesigned.

Table 4.3: The primers designed by Primer Express and used for SYBR green RT-PCR to measure the expression of *R. equi* plasmid pathogenicity island genes (pVAPA_*vapA*, pVAPA_*vapC*, pVAPA_*vapF*, pVAPA_0560).

Target gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
pVAPA_*vapA*	ataccgccgggcaagag	tagacgaccgcgctgatg
pVAPA_*vapC*	tcgtccatccaaatcgatagc	tcccaaggccaagaagca
pVAPA_*vapF*	ttttagcgcggtgttttataacc	gaaagtcattcccccatcga
pVAPA_0560	ccgagtcgggcattgaag	cccatcgctactcatatgaatcc

4.2.9.4. qPCR reactions using *Power* SYBR Green PCR Master Mix

The *Power* SYBR Green PCR Master Mix was allowed to thaw completely and mixed before use. The PCR reagents were prepared in an Eppendorf tube and mixed by scaling the volumes listed below to the desired number of PCR reactions. Extra volume was included to account for pipetting errors.

Power SYBR Green PCR Master Mix (2×)	12.5 µL
Reverse Primer (10 uM)	0.75 µL
Forward Primer (10 uM)	0.75 µL
cDNA (diluted 1:100)	10 µL
Nuclease-free Water	1 µL
Total	25 µL

For convenience, the former three components (SYBR green, reverse and forward primers) were first combined and distributed 15 µL each into the 96-wells plate according to the layout. The latter two components (cDNA and water) were added directly into the wells and mixed. To seal the plate, optical adhesive tape was applied on it completely. The plate was then centrifuged briefly to remove the bubbles. For every primer pair the best concentrations was worked out by trying varying concentrations of the primers (data not shown). Endogenous control i.e. 16S rRNA was used as a housekeeping gene. The general thermal-cycling conditions for Power SYBR Green PCR Master Mix used were the following:

	Enzyme Activation	PCR Cycles (40)	
		Denature	Anneal/Extend
Time	10 min	15 sec	1 min
Temperature	95°C	95°C	60°C

The reaction plate was loaded into the instrument and then the programme started. Following the completion of the run, the data was collected.

4.2.9.5. Data analysis and presentation

After the completion of thermal cycles, the retrieved data was analysed. The threshold level was set to 1.0 and all the C_t values (averages) were transferred to Excel. The ΔC_t was calculated by subtracting C_t sample from C_t endogenous control. The $\Delta\Delta C_t$ was calculated by subtracting ΔC_t sample from ΔC_t reference. Thereby, $2^{-\Delta\Delta Ct}$. was determined which represents the fold-change value. For negative values the reciprocal of that value was taken to get the fold-change. A bar graph was plotted from these values taking fold-change values along the y-axis.

4.2.10. *In vitro/in vivo* gene expression of *R. equi*

For measuring *in vitro* expression of *R. equi* virulence genes at various temperatures, bacteria were cultured in LB medium at 37, 36, 34, 32, 30 and 22ºC. The RNA was then extracted as described above (section 4.2.9.1. and 4.2.9.2.). Comparative gene expression was calculated using ΔΔCt method (Kakuda *et al*., 2014) in terms of fold change for the specified genes.

For the *in vivo* experiments, infections were performed in 175 cm^2 cell culture flasks as for macrophages. The bacteria were released from the infected cells by 50 mL ice-cold 4M GTC (4 M guanidine thiocyanate, 25 mM tri-sodium citrate, 0.5% sodium N-lauryl sarcosine, 0.1 M 2-mercaptoethanol and 0.5% Tween 80)(Ren and Prescott, 2003). These flasks were then placed on ice and gently shook for 5 min before transferring the lysate into centrifuge tubes. After centrifugation at 2500 ×g for 30 min, most of the supernatant was removed and the pellet was resuspended in the minute leftover supernatant (Ren and Prescott, 2003). This was then moved into a cold Eppendorf tube. From this bacteria suspension, RNA was isolated as described above (section 4.2.9.1. and 4.2.9.2.).

Parallel infections in macrophages (J774A.1) and *Acanthamoeba* (MN-7) were carried out in flasks to have a greater number of cells. The upregulation of virulence genes

vapA, *vapC* and *vapF* of *R. equi* in infected cells at 24h of infection was measured by lysing these cells with GTC as described above for the isolation of total RNA. The 16S rRNA gene was used as housekeeping gene.

4.2.11. Strategies for increasing bacterial internalization

Different strategies were tried to increase the internalization of bacteria and to increase the number of bacteria at t=0 of infection. Different MOIs were used to see any significant increase in internalization of *R. equi* by *Acanthamoeba*. MOIs of 100:1 and 1000:1 were tried.

To observe the effect of changing the medium/depriving *Acanthamoeba* of the nutrients, the infection the medium (AX2+) was replaced by PBS to see any increase in phagocytosis of *R. equi*. This was followed by the infection as usual. To see the effect of lowering the temperature on phagocytosis of *R. equi*, temperature down to 18ºC was used. The plates were left at this temperature after infecting amoeba cells with *R. equi*. The combined effect of changing medium and lowering temperature was also studied. For this purpose the AX2+ medium was replaced by PBS and after infection the plates were placed at 18ºC.

For preventing phagosomal acidification, *Acanthamoeba* cells were pre-treated with ammonium chloride (NH_4Cl) to increase the survival of *R. equi* inside the phagosome and as a result overall number of bacteria at t=0. Ammonium chloride solution at a concentration of 25 mM was used in the AX2+ and the cells in 24-wells plate were covered with this for 1h to penetrate the cells. This was then followed be removal of the medium and infection with *R. equi* (Akya *et al*., 2009).

Another strategy used to increase the number of bacteria at t=0 in *Acanthamoeba* cells, aimed at stressing the cells with acidic pH. A low pH of 5.0 was used for this purpose. The *Acanthamoeba* cells were left in AX2+ medium (pH 5.0) for overnight. The next day the medium was removed followed by infection assay as normal (Axelsson-Olsson *et al*., 2010).

4.3. Results

4.3.1. Growth curves of *R. equi*

The growth curves in BHI and AX2+ with and without vancomycin showed that *R. equi* could grow in AX2+ although the maximum growth level was not as good as in BHI. Also, vancomycin showed good efficacy as bacteria were unable to grow in the presence of vancomycin (Figure 4.6).

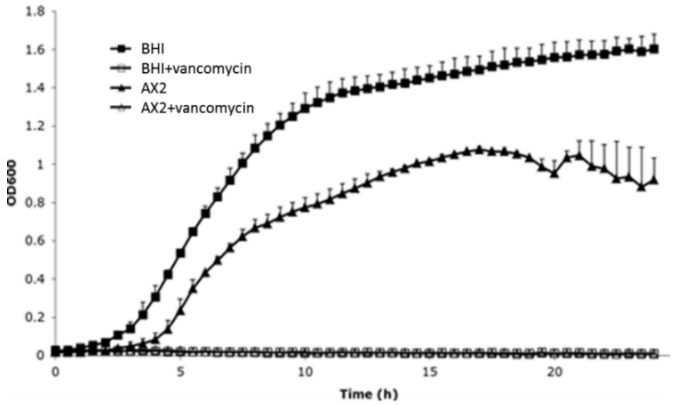


Figure 4.6: Growth curves of *R. equi* 103^{+} in BHI and AX2+ medium at 37ºC with and without vancomycin (5 µg/mL). The data represents mean±SD of three independent experiments.

4.3.2. Setting up of experimental conditions

4.3.2.2. Effect of centrifugation on sedimentation of bacteria

To observe the effect of centrifugation on the sedimentation to increase contact with the cells, the 24-well plate was spun at 900 rpm for 3 min. Centrifugation proved very useful in settling down the bacteria after infection of *Acanthamoeba* with bacteria (Figure 4.7).

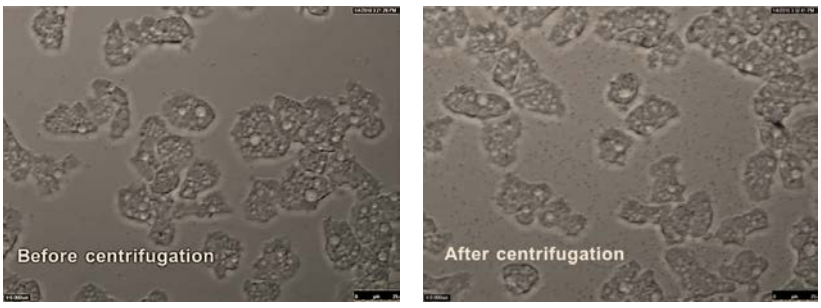
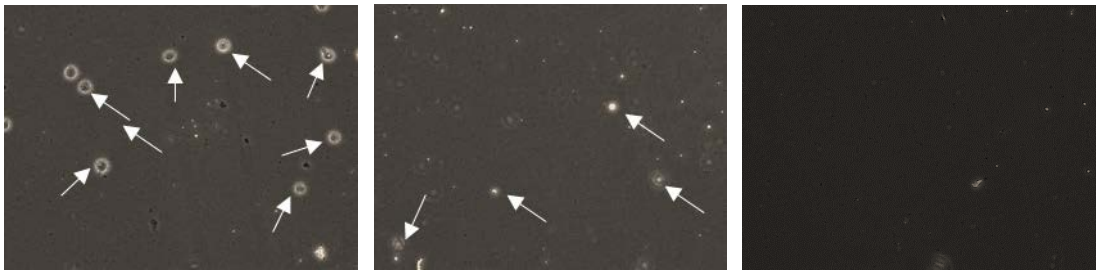


Figure 4.7: Effect of centrifugation on sedimentation of bacteria. Centrifugation had definite effect on sedimenting bacteria down to the amoebal cell monolayer for good contact and thus increasing their chance of being engulfed by the cells.

4.3.2.3. Effect of triton on lysis of *Acanthamoeba* cells

Three different concentrations of triton (0.01, 0.5 and 0.1%) were used in an attempt to achieve complete lysis of *Acanthamoeba* (MN-7) cells. The 0.1% triton lysed the cells very quickly while 0.01% concentration could not lyse the cells completely even after vortexing the cells (Figure 4.8). The 0.1% triton seemed more acceptable as it lysed majority of the cells completely over the period of 5 min while remaining partially lysed cells were fully lysed after brief vortexing. Vortexing helped avoid using higher concentration of triton to achieve complete lysis.



0.01% 0.05% 0.1%

Figure 4.8: Lysis of *Acanthamoeba* MN-7 cells with various concentrations of triton X-100 showing cells lysed after vortexing the cells. *Acanthamoeba* cells were exposed to three different concentrations of triton X-100 (0.01, 0.05 and 0.1%) for 5 min followed by 5× dilution with PBS and brief vortexing. The 0.01% concentration was the most ineffective and many intact amoeba cells can still be seen (arrows). Fewer intact or incompletely lysed cells were seen in 0.05% concentration (arrows) while no intact cells were seen in 0.1% concentration of triton.

4.3.3. Interaction of *R. equi* with macrophages

4.3.3.1. Optimization of infection assay in macrophage cell lines

The intracellular survival of *R. equi* was tested primarily in J774A.1 macrophages and the findings were consolidated in another macrophage cell line (RAW264.7) as well. The method used was simple and involved fewer steps as the bacteria were directly added onto the macrophages and they were settled down by centrifugation to increase their contact with macrophages. In both the cell lines, the internalization of bacteria was fairly high. There was a growing trend for plasmid-bearing strain (103^{+}) by 24h with a major boost by 48h while the plasmid-cured strain (103^{-}) was unable to sustain its existence for longer and continuously declined in number by 48h (Figure 4.9). The uninfected control cells were found to have very low levels of nitrite, indicating that the cells were in normal inactivated state. However, the infected cells demonstrated

higher levels of nitrite as a result of bacterial invasion. Microscopically, at 48h there was no extensive destruction of the macrophages and they looked intact. The nitrite levels in the cells were also fairly low.

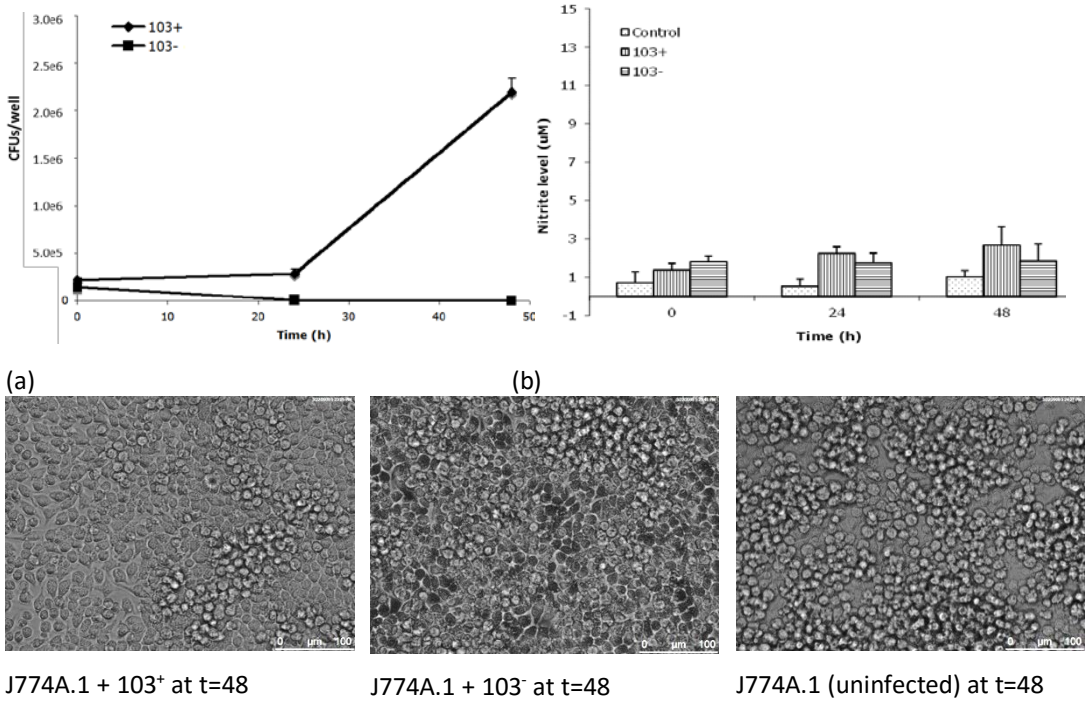


Figure 4.9: The survival of *R. equi* WT plasmid-bearing (103^+) and plasmid-cured (103^-) in murine J774A.1 macrophages at 37°C. There was proliferation of 103^+ bacteria by 24h and by 48h the bacterial number boosted sharply. The plasmid-cured bacteria, however, were unable to survive and continuously declined (a). The control expressed very low nitrite levels indicating that the macrophages were normal and not in an activated state. The treated macrophages, however, expressed slightly higher levels of nitrite as a result of infection (b). The bottom panel shows J774A.1 macrophages infected with *R. equi* 103^+ (left) and the plasmid-cured 103^- (middle) 48 h post-infection in comparison with the uninfected control cells (right). There was no major destruction of cells and the cells looked intact which means proliferation of *R. equi* 103^+ without macrophage lysis.

Infection in RAW264.7 macrophages also exhibited similar growth pattern of *R. equi* as seen in J774A.1 macrophages i.e. excessive proliferation of plasmid-bearing 103^+ bacteria in macrophages without significant cell lysis (Figure 4.10). Moreover, the plasmid-cured 103^- bacteria could not survive longer and gradually declined. The nitrite level of uninfected macrophages was low (indicating a normal inactivated state of macrophages), like J774.A1 while the infected cells had higher nitrite level mainly those infected by 103^+, indicating a more aggressive response of the RAW264.7 macrophages against the *vap*+ bacteria than J774.A1 macrophages. These findings are

comparable with those in J774A.1 macrophages although the RAW264.7 macrophages reacted more violently at 48h.

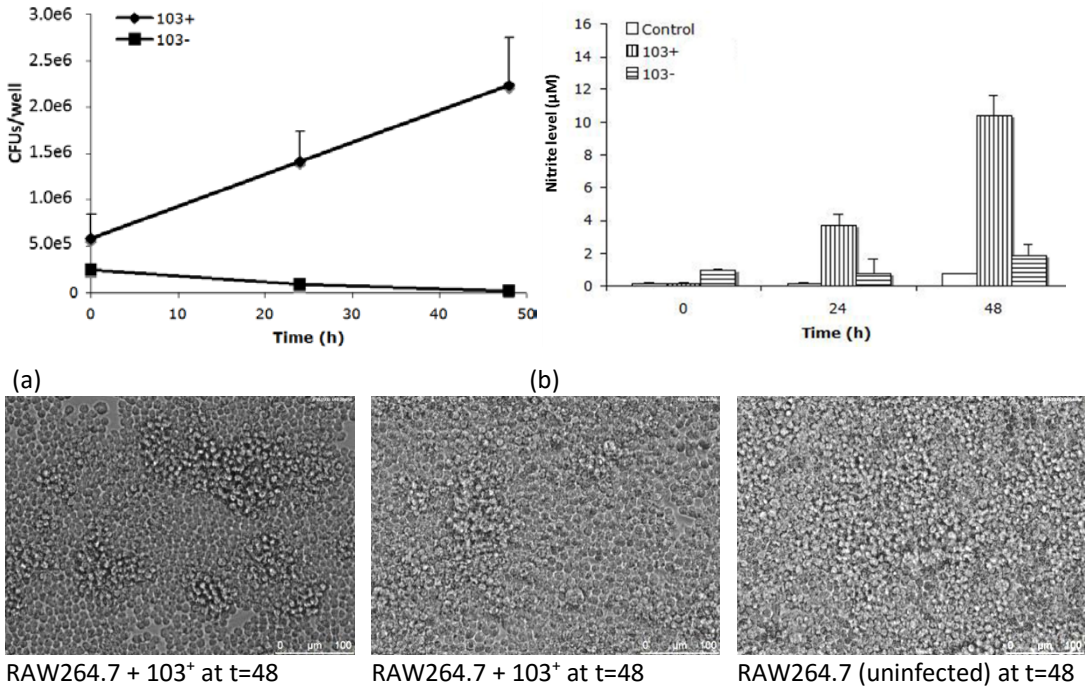


Figure 4.10: The survival of *R. equi* WT plasmid-bearing (103^+) and plasmid-cured (103^-) in murine RAW264.7 macrophages. A similar trend was observed as in J774A.1. There was proliferation of 103^+ bacteria by 24h and by 48h the bacterial number boosted sharply. The plasmid-cured bacteria, however, were unable to survive and continuously declined (a). The control expressed very low nitrite levels indicating that the macrophages were normal and not in an activated state. The treated macrophages, however, expressed higher levels especially with 103^+ at 48h probably because of more aggressive response of macrophages (b). The bottom panel shows RAW264.7 macrophages infected with *R. equi* 103^+ (left) and the plasmid-cured 103^- (middle) 48 h post-infection in comparison with the uninfected control cells (right). There was no major destruction of cells and the cells looked intact which means proliferation of *R. equi* 103^+ without macrophage lysis.

4.3.3.2. Consolidation of infection using other WT and mutant *R. equi* strains

Further consolidation of the infection assay for studying the intracellular survival of *R. equi* in macrophages was made using other WT strains including RE1 (equine isolate), 1571 (bovine isolate) and 1593 (porcine isolate). All the isolates had similar proliferation trend in J774A.1 macrophages (Figure 4.11).

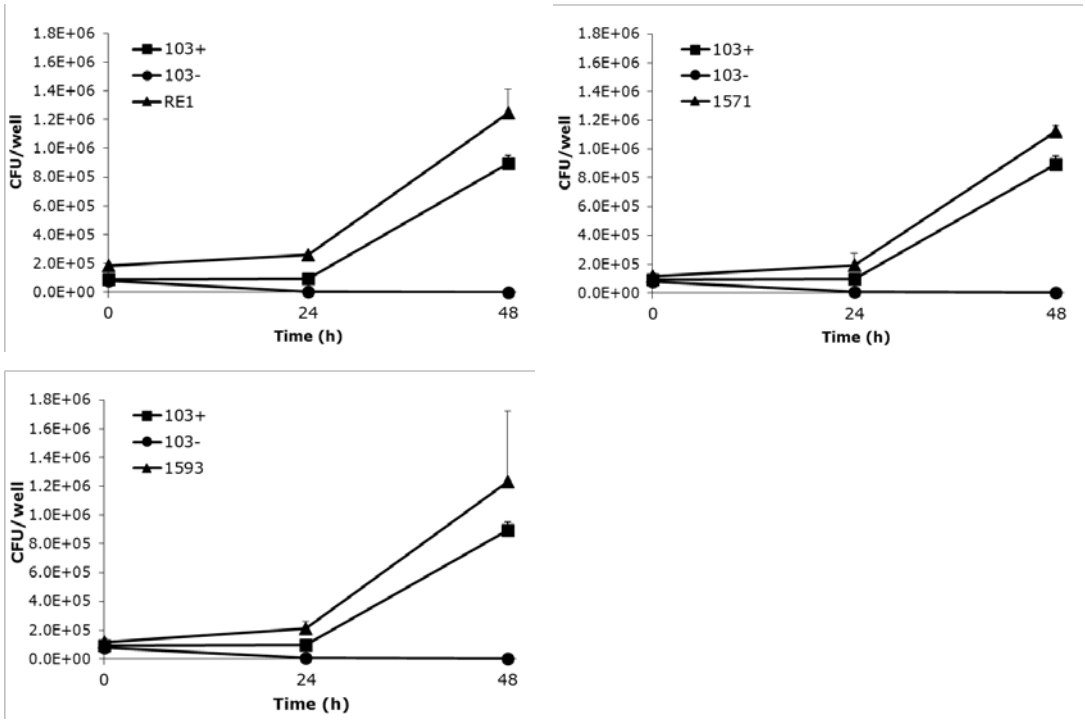
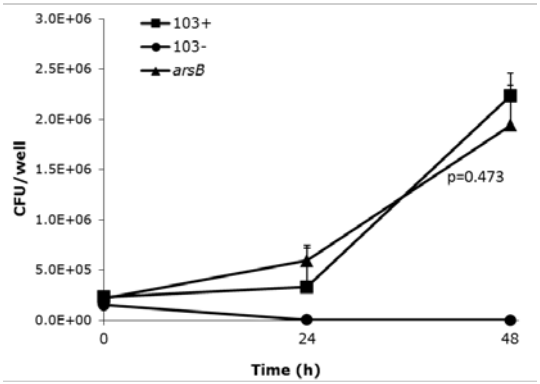


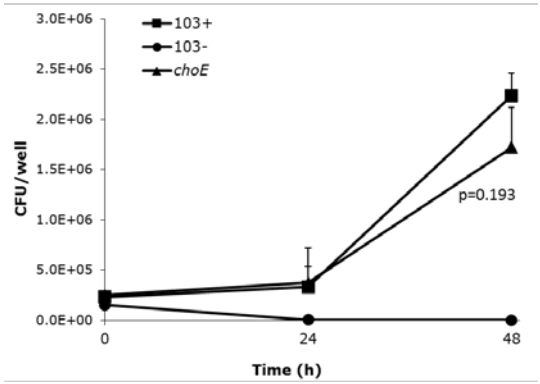
Figure 4.11: The survival of *R. equi* wildtypes 103^+, RE1, 1571, 1593 and plasmid-cured 103^- in murine macrophages (J774A.1). All the plasmid-bearing strains showed the similar proliferation trend in macrophages while the plasmid-cured strain 103^- was unable to grow. The data represents mean±SE of three independent experiments.

To further confirm the validity of infection assay for *R. equi* and macrophages, some of the *R. equi* mutant strains were tested (Figure 4.12). These included mutants strains with mutation in *arsB*, *choE*, *supAB*, *mce1*, *chori* and *ureA* genes. Out of these, the mutants *mce1* and *chori* are known to have an effect of mutation on the intracellular survival in macrophages which was also consistent with the results of infection assays in macrophages with these mutants. Although the *chori* mutant was able to show some proliferation but the survival was considerably reduced as compared to the wildtype (103^+) strain, which shows an effect of mutation on intracellular survival. These results further validate the infection assay used for monitoring of intra-macrophagal survival of *R. equi*.

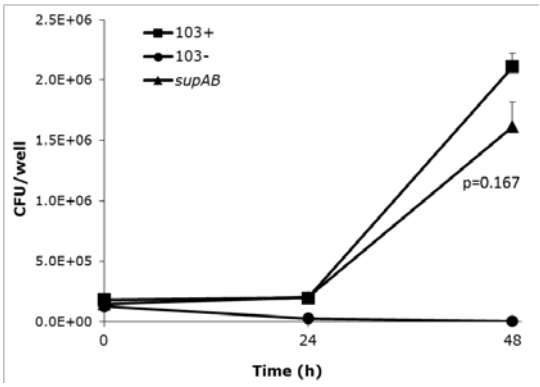
Overall, the results of infection assays indicate survival and proliferation capability of *R. equi* in macrophages in plasmid-dependent manner without major cell destruction.



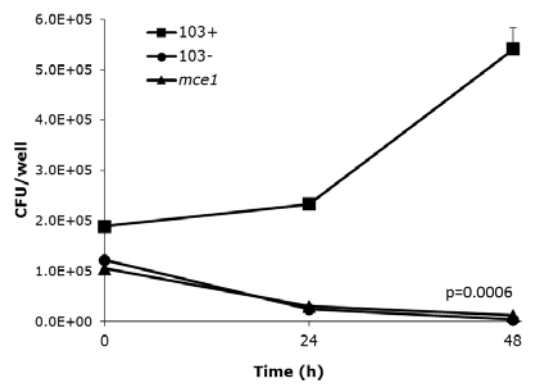
R. equi arsB (n=3)



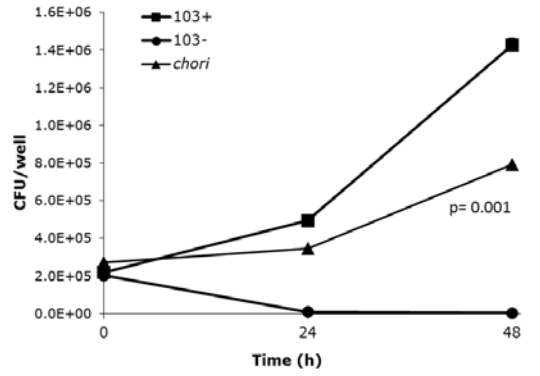
R. equi choE (n=3)



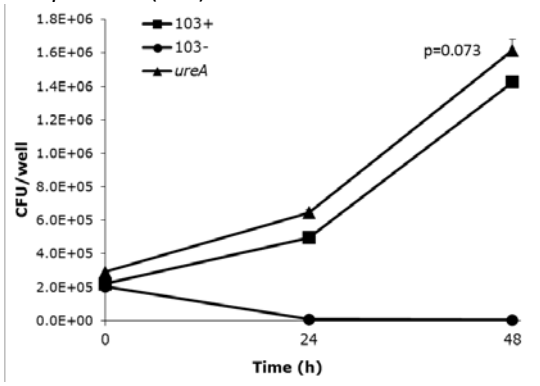
R. equi supAB (n=2)



R. equi mce1 (n=3)



R. equi chori (n=2)



R. equi ureA (n=2)

Figure 4.12: Intracellular survival of *R. equi* mutant strains (*arsB*, *choE*, *supAB*, *mce1*, *chori* and *ureA*) of in murine macrophages J774A.1 along with WT strain 103^{+} and the isogenic plasmid-cured strain 103^{-}. The *mce1* and *chori* mutants showed an effect of mutation. Data represents mean±SE of "n" experiments as shown below respective graph. In each set of experiment, the *R. equi* 103^{+} and 103^{-} strains were included as positive and negative controls, respectively. The comparison was made between the test and the control at t=48 using the GraphPad Student's t-test at 95% confidence level.

4.3.4. Interaction of *R. equi* with *Acanthamoeba*

A similar method was used for studying the intracellular survival of *R. equi* in *Acanthamoeba* (MN-7), however, modifications were employed in order to take care of any cells detached at 37°C (section 4.2.8.1) which intended at linking number of

bacteria recovered with the number of amoeba cells at any time point for more accurate monitoring of intracellular survival of bacteria. Apart from this, attempts were made to improve the uptake of *R. equi* by the amoebae.

4.3.4.1. Setting up of conditions for infection of *Acanthamoeba* with *R. equi*

4.3.4.1.1. Increasing multiplicity of infection (MOI)

The effect of increasing the multiplicity of infection was accessed by infecting *Acanthamoeba* Neff cells with *R. equi* at the MOI=100:1 with parallel infection at the MOI=1000:1. However, the results indicated that increasing the MOI from 100:1 to 1000:1 had no significant effect (p=0.162) on internalization of bacteria (Figure 4.13). Therefore, for all the experiments MOI=100:1 was used.

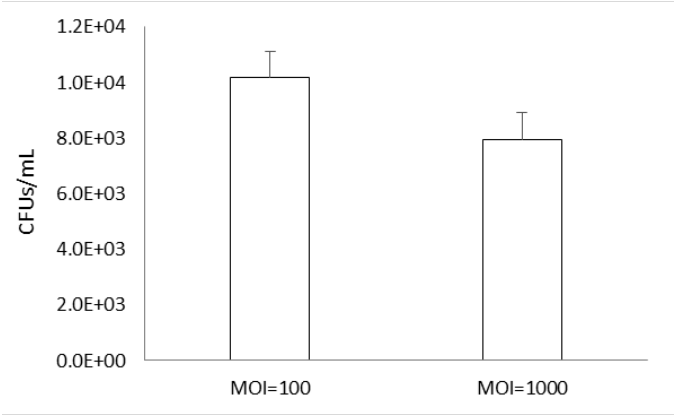


Figure 4.13: Effect of MOI (100:1 and 1000:1) on the uptake of *R. equi* by *Acanthamoeba* (MN-7). Instead of increase in uptake of bacteria at MOI=1000:1 there was rather a decrease (p=0.162 at 95% confidence level; using the GraphPad Student's t-test). The data represents mean ± SE of three independent experiments.

4.3.4.1.2. Changing the medium / depriving *Acanthamoeba* of the nutrients

Depriving amoebae of nutrients overnight to make them "hungry", resulted in reduced uptake of *R. equi* by *Acanthamoeba* cells (p=0.7127 at 95% confidence level) (Figure 4.14). Therefore, nutrient deprivation does not have a significant effect on increasing uptake of bacteria by *Acanthamoeba* (MN-7).

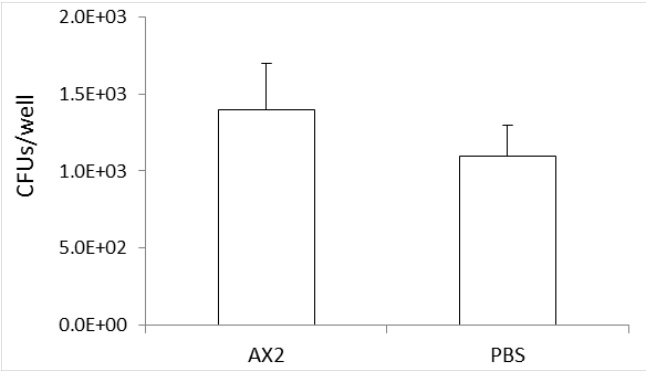


Figure 4.14: The effect of replacing growth medium (AX2) of *Acanthamoeba* (MN-7) with the PBS to deprive them of the nutrients by making them "hungry" had rather a negative impact on the uptake of bacteria (p=0.7127 at 95% confidence level; using the GraphPad Student's t-test). The data represents mean±SE of two independent experiments.

4.3.4.1.3. Lowering the temperature

Parallel infections at RT (23°C) and at lower temperature (18°C) were conducted to find out if the uptake of *R. equi* by *Acanthamoeba* could be increased. However, lowering the temperature did not have a significant effect (p=0.5636 at 95% confidence level) on increasing the uptake of *R. equi* by *Acanthamoeba* MN-7 (Figure 4.15). Therefore, it does not matter whether infections *Acanthamoeba* (MN-7) by *R. equi* are carried out at RT or at lower temperature.

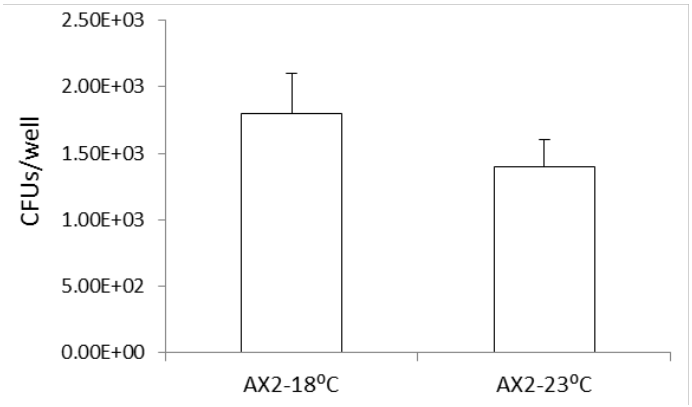


Figure 4.15: The effect of lowering the temperature on the uptake of bacteria by *Acanthamoeba* (MN-7). The infection was carried out at 18°C parallel with infection at 23°C but there was not significant increase in uptake of bacteria (p=0.5636 at 95% confidence level; using the GraphPad Student's t-test). The data represents mean±SE of two independent experiments.

4.3.4.2. Survival of *R. equi* in *Acanthamoeba*-role of plasmid and temperature

At virulence genes **activated temperature (37°C)**, *R. equi* 103^+ showed better survival than the plasmid-cured 103^- strain. During the 48h long infection the difference between the number of bacteria of 103^+ and 103^- was significant at 24h which declined significantly by 48h (Figure 4.16).

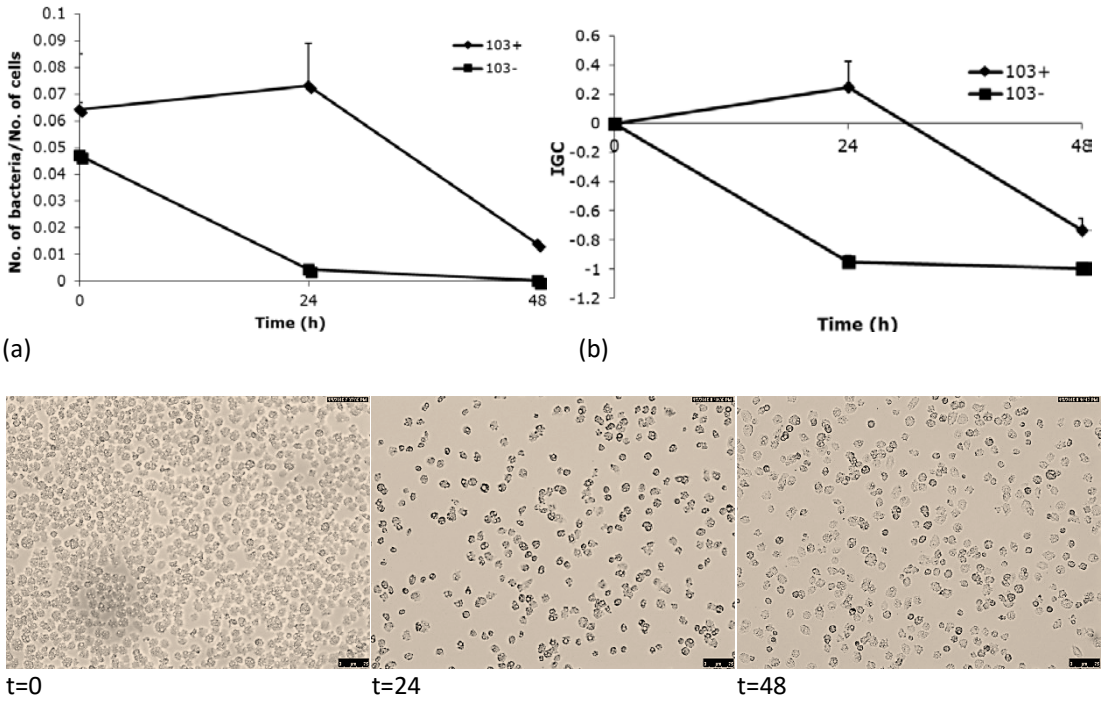


Figure 4.16: Intracellular survival of *R. equi* in *Acanthamoeba* MN-7 at virulence activated temperature 37ºC. The bacteria manifested survival in plasmid and temperature dependent manner as only plasmid-bearing strain (103^+) showed survival and some proliferation but the plasmid-cured strain (103^-) could not survive (a) which is more evident in terms of IGC (b). Amoebae had tendency of detaching the surface of 24-well plate so a drop of cells was seen by 24h but no further detachment was observed after that (c). Cells are from control wells at t=0, 24 and 48h. The data represents mean±SE of three independent experiments.

At virulence genes **deactivated temperature (22°C)**, both the *R. equi* strains showed inability to survive. This is as expected because of failure of virulence genes to be activated at this low temperature (Figure 4.17).

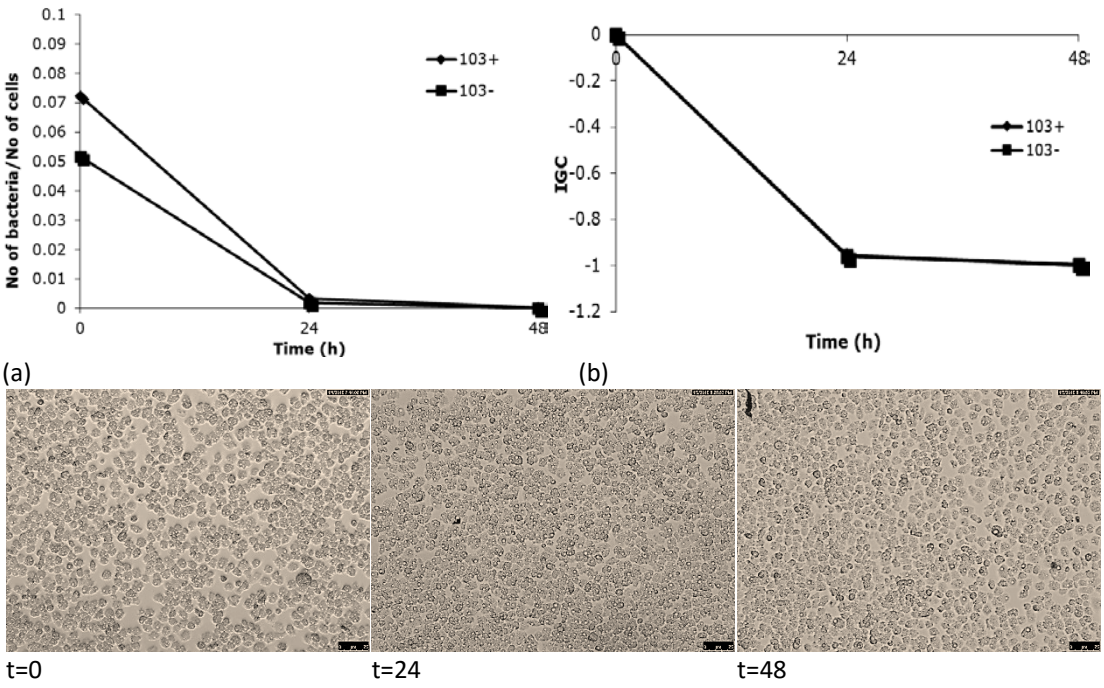


Figure 4.17: Intracellular survival of *R. equi* in *Acanthamoeba* MN-7 at virulence deactivated temperature 22ºC. The bacteria manifested showed no survival in neither plasmid nor in temperature dependent manner as both the plasmid-bearing strain (103^{+}) as well as plasmid-cured strains (103^{-}) could not survive (a) which is more evident in terms of IGC (b). Amoebae did not had tendency of detaching the surface of 24-well plate so no drop of cells was seen (c) Cells are from control wells at t=0, 24 and 48h. The data represents mean±SE of three independent experiments.

4.3.4.2.1. Attempts at improving the internalization of *R. equi* by *Acanthamoeba*

Attempts were made to enhance the uptake of *R. equi* by *Acanthamoeba* MN-7 cells; these included prevention of phagosomal acidification and use acid stress.

4.3.4.2.1.1. Preventing phagosomal acidification

Pre-treating *Acanthamoeba* (MN-7) cells with ammonium chloride (25 mM) for 1h improved internalization of *R. equi* (as compared with infection without any pre-treatment; Figures 4.16 and 4.17), however, the treatment affected the long term survival of bacteria as shown with full infections up to 48h (Figure 4.18). The bacteria could not survive for longer. This was inconsistent with the normal intracellular survival pattern of *R. equi* in *Acanthamoeba* (Figure 4.16). It indicates that although the ammonium chloride treatment help increase the uptake of *R. equi* by *Acanthamoeba*, it also impacts negatively the long term intracellular survival of

bacteria. Therefore, this methods is not suitable for studying the survival of *R. equi* in *Acanthamoeba* and was thus not used for infection assays any further.

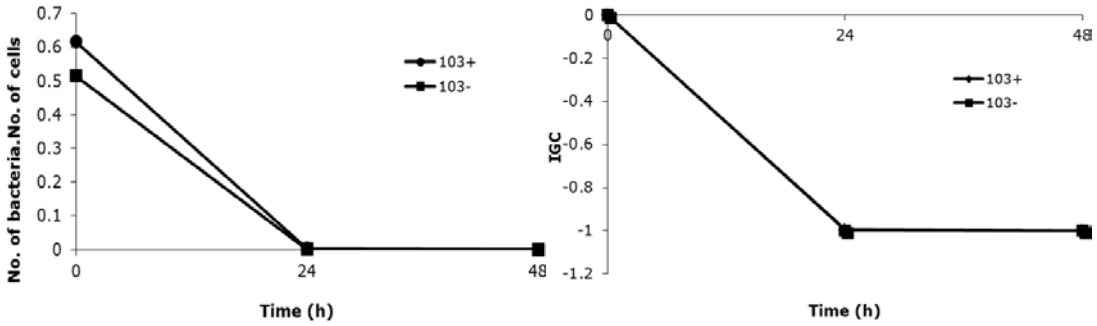


Figure 4.18: Effect of ammonium chloride (25 mM) pre-treatment on uptake of *R. equi* by *Acanthamoeba* (MN-7). Although there was an increase in the uptake of bacteria but the treatment had an effect of long term survival of bacteria. The data represents mean±SE of three independent experiments.

4.3.4.2.1.2. Acid stress

Acid stress (pH 5.0) also improved internalization of *R. equi* by *Acanthamoeba* MN-7 cells (Figure 4.19) as compared with infection without any pre-treatment (Figures 4.16 and 4.17), however, like ammonium chloride treatment (section 4.3.4.2.1.1.), the bacteria could not survive longer intracellularly. This is not the usual behaviour of *Acanthamoeba* towards *R. equi* (Figure 5.16) and it indicates an impact of acid stress on the normal response of amoeba cells towards these bacteria, therefore, the acid pre-treatment to enhance the uptake of bacteria during infection assay was not use any further.

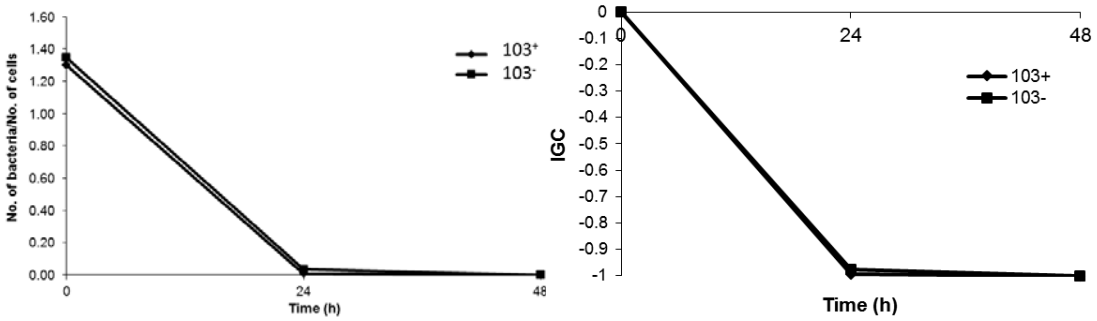


Figure 4.19: Effect of acid stress (lower pH) on uptake of *R. equi* by *Acanthamoeba* (MN-7). Amoebae were exposed to low pH overnight before infecting them with *R. equi*. Although there was a significant increase in uptake of bacteria but the treatment had an effect of long term survival of bacteria. The data represents mean±SE of three independent experiments.

4.3.4.3. Consensus temperature for *Acanthamoeba* infection with *R. equi*

A clear view of the intracellular survival pattern of *R. equi* in *Acanthamoeba* was obtained at 37°C (Figure 4.17), however, to further consolidate these findings attempts were made to work out a more reasonable lower temperature which is more favourable for amoebae without suffering detachment of cells but at the same time *R. equi* virulence genes are still activated. For this purpose a titration curve for *R. equi* virulence genes activation was required against various temperatures. For this purpose, *in vitro* expression profile of *R. equi* PI was studied at various temperatures.

4.3.4.3.1. *In vitro* Expression profile of *R. equi* PI genes

RT-PCR was used to study the expression levels of *R. equi* PI genes over a range of temperatures (22, 30, 32, 34, 36 and 37°C). Four genes from the PI were used including pVAPA_*vapA*, pVAPA_*vapC*, pVAPA_*vapF* and pVAPA_0056. The expression of virulence genes was found to be very precisely regulated by the temperature (Figure 4.20).

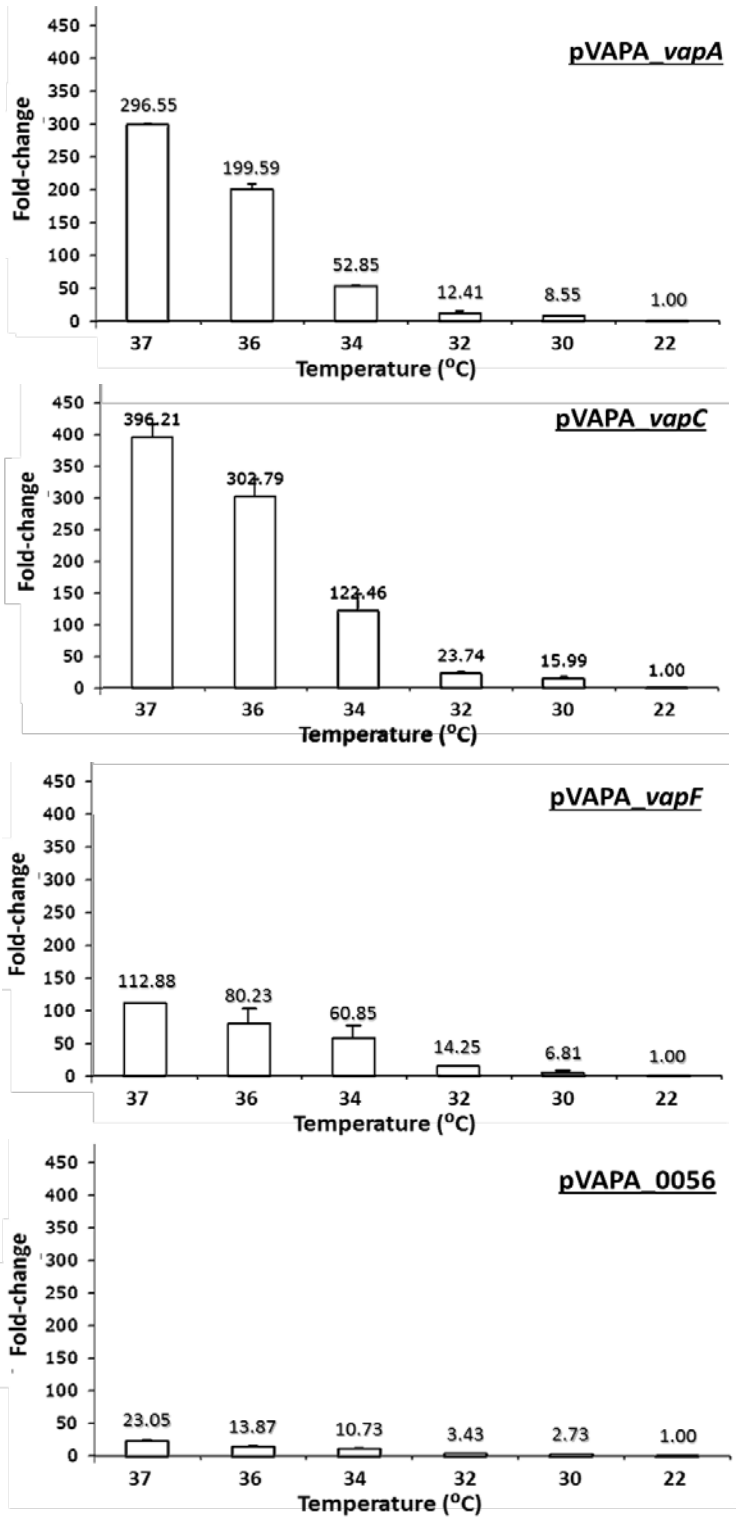


Figure 4.20: *In vitro* expression of *R. equi* plasmid virulence genes *vapA* (a), *vapC* (b), *vapF* (c) and 0560 (d) at 37ºC, 36ºC, 34ºC, 32ºC and 30ºC relative to expression at 22ºC (fold-change). These experiments were carried out to find the precise regulation of virulence genes expression as a function of temperature to find a reasonable temperature which is good for growth of *Acanthamoeba* (MN-7) but still has plasmid genes expressed to show their effect. The 16S rRNA gene was used as housekeeping control. *R. equi* (103^{+}) were grown in LB medium at the designated temperatures followed by RNA isolation and RT-PCR. The data represents mean±SE for three independent experiments.

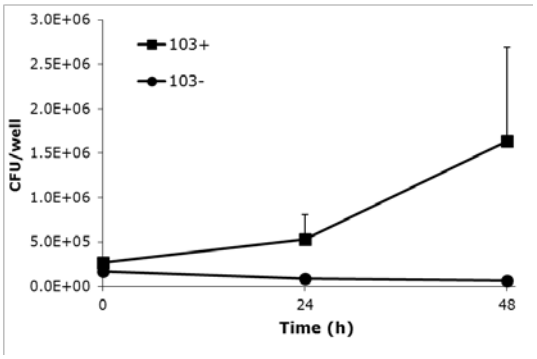
These expression profiles of *R. equi* virulence genes provided very useful information as these indicated precise correlation of the expression of these genes with changing temperature. This information helped choose a consensus temperature which is lowest enough for good growth and adhesion of amoeba cells with the 24-well plate but higher enough for adequate expression of *R. equi* virulence genes for proliferation in macrophages.

4.3.4.4. Intracellular survival of *R. equi* at 34°C

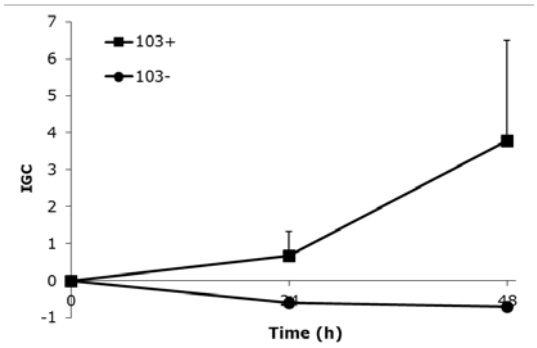
For the infection experiments with *R. equi* and *Acanthamoeba*, 34°C was initially selected as it appeared to have adequate expression of virulence genes. Moreover, amoebae did not suffer detachment at this temperature. However, to confirm whether this expression of virulence genes was able to support the intracellular survival of *R. equi* in macrophages, infections were performed at 34°C. The results are described below.

4.3.4.4.1. Survival in macrophages at 34°C

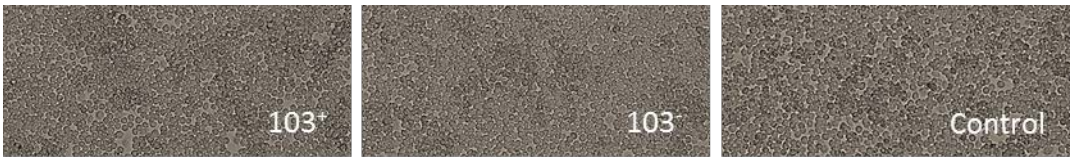
Before infections in *Acanthamoeba* with *R. equi* were carried out, infections in macrophages were performed to see if practically the expression of virulence genes at 34°C (as seen in *in vitro* expression experiments; section 4.3.4.3.1.) would be able to sustain survival of *R. equi* before actually carrying out infection in *Acanthamoeba* at this temperature. The results of the infection experiments indicated that *R. equi* were able to survive in plasmid-dependent manner at this temperatures (Figure 4.21-a1,a2,a3). Parallel infections were also carried out at 25°C (as 22°C was not reachable by the incubator) as control to see if survival in macrophages is also temperature dependent, however, these infections were inconclusive because of inability of macrophages to sustain their morphology and physiology at this low temperature and as a consequence apparently both the 103^{+} and 103^{-} *R. equi* showed proliferation (Figure 4.21-b1,b2,b3).



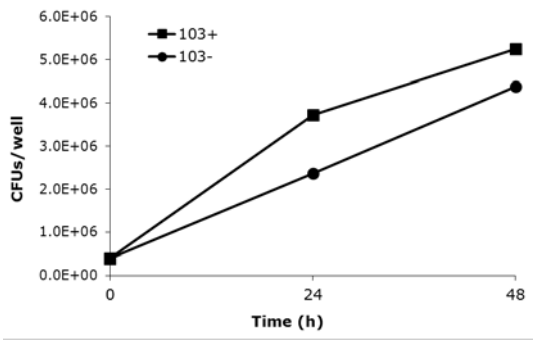
(a1)



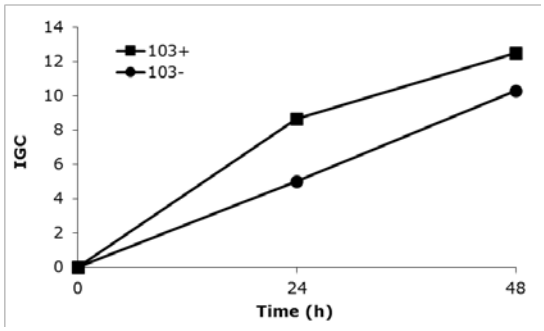
(a2)



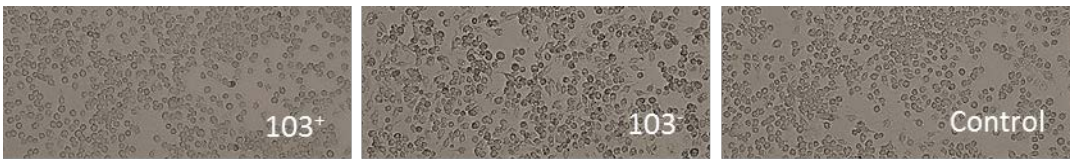
(a3)



(b1)



(b2)



(b3)

Figure 4.21: Intracellular survival of *R. equi* in murine macrophages (J774A.1) at 34ºC (a1, a2, a3) with infection at 22°C (b1, b2, b3) as control at deactivated temperature. Infection assay at 34ºC was carried out as normal using the plasmid-bearing (103^+) as well as plasmid-cured (103^-) strains of *R. equi*. The experiment was aimed at confirmation of adequacy of 34°C temperature to sustain survival of *R. equi* in macrophages. *R. equi* showed survival in macrophages in plasmid and temperature dependent manner (a1 and a2) as in case of infection at 37°C. The bottom panel (a3) shows J774.A1 macrophages infected with *R. equi* 103^+ and 103^- in comparison with non-infected control at 48h post-infection. The cells looked healthy and proliferating as normal. However, the cells at 25°C did not look normal at this low temperature probably because this was too low temperature for them to sustain their structural and functional integrity. Therefore, these findings are not valid.

4.3.4.4.2. Survival of *R. equi* in *Acanthamoeba* at 34ºC

Acanthamoeba MN-7 infections were conducted at 34ºC. The WT with plasmid (103^+) slightly grew better than plasmid-cured *R. equi* (Figure 4.22). These results supported the findings made with infection experiment at 37°C (section 4.3.4.2.).

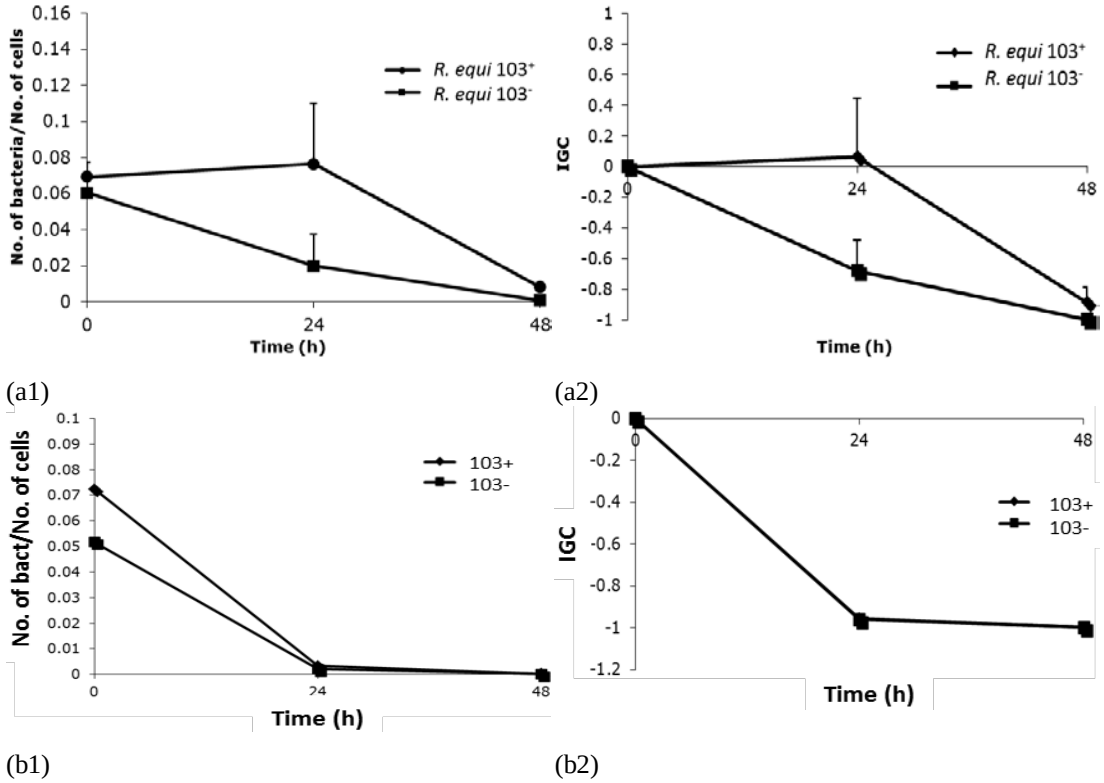


Figure 4.22: Intracellular survival of *R. equi* in *Acanthamoeba* MN-7 at 34ºC (a1, a2) while infections at 22°C (b1, b2) were carried out as control infections at virulence deactivated temperature. Infections at 34°C were performed as amoebae don't detach at this temperature and *R. equi* plasmid genes are also activated at this temperature. The results were not different from those of infections in *Acanthamoeba* at 37°C. *R. equi* showed survival in amoeba cells in plasmid and temperature dependent manner (a1) which is clearer in terms of IGC (a2). *R. equi* at deactivated temperature did not show survival even in plasmid-bearing 103^+ strain (b1 and b2). The data represents mean±SE of three independent experiments.

4.3.4.5. *In vivo* gene expression of *R. equi* in macrophages and *Acanthamoeba*

The expression of the three plasmid virulence genes *vapA*, *vapC* and *vapF* of *R. equi* was determined after infection of macrophages as well as *Acanthamoeba*. The cells were infected and the bacteria were recovered at 0h and then at 24h post-infection. The RNA of bacteria was extracted and the expression of these genes at 24h was determined relative to 0h. The data from macrophage experiment showed ~18 fold

increase in expression of both *vapA* and *vapC* while 5 fold for *vapF* (Figure 4.23) which shows the importance of *vapA* and *vapC* in survival of *R. equi* in macrophages.

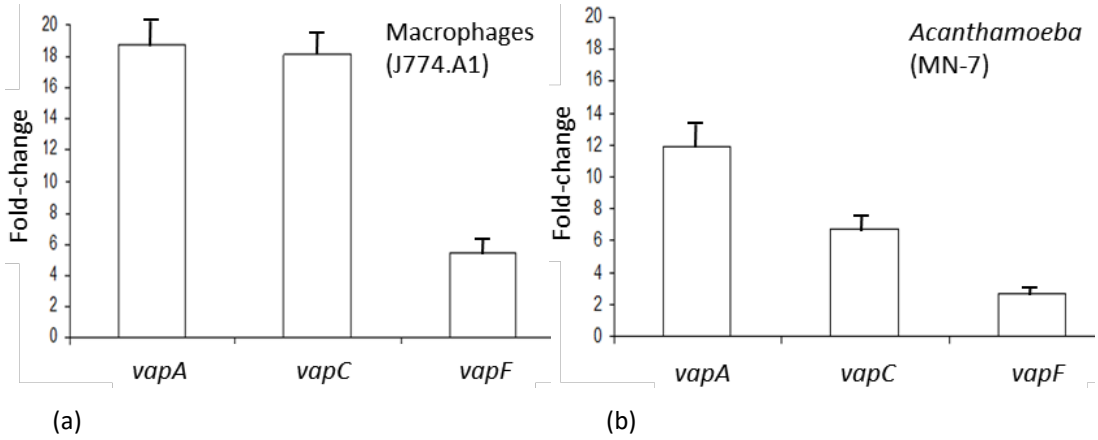


Figure 4.23: Intracellular expression of *R. equi vap* PI genes in J774.A1 macrophages (a) and *Acanthamoeba* MN-7 (b) at 34°C, 24h post-infection indicating that *vapA* and *vapC* are mainly activated in macrophages during intracellular survival of *R. equi* 103^{+} (nearly 18 times). The data represents mean±SE of two independent experiments.

In case of *Acanthamoeba*, *vapA* was the maximum expressed virulence gene (12 fold) followed by *vapC* (6 fold) and *vapF* (3 fold). This shows that *vapA* is more important in survival of *R. equi* in *Acanthamoeba* as compared to both *vapA* and *vapC* in case of macrophages.

The findings of survival of *R. equi* at 34ºC in a plasmid-dependant manner are promising and strengthen the hypothesis that "intra-amoebal survival of *R. equi* may be pre-adaptive for their existence as pathogens by surviving in macrophages".

Overall, the findings of this study indicate that the *R. equi* pathogenicity island (PI) on plasmid plays central role in their survival in both macrophages and *Acanthamoeba* (MN-7) and is further supported by the observation that intra-amoebal survival of *R. equi* is temperature dependent. The survival strategies appear to involve common genes as demonstrated by upregulation of similar PI virulence genes (*vapA*, *vapC* and *vapF*) during survival in macrophages and *Acanthamoeba*. Therefore, these observations appear to support the role of amoebae in pre-adaptation of *R. equi* as pathogen in macrophages.

4.4. Discussion

This chapter investigated the intracellular survival strategies of the emerging human pathogen *R. equi* in macrophages as well as in *Acanthamoeba*. Although *R. equi* closely resembles mycobacteria, its genome is very different and interesting from other bacteria. The genome of *R. equi*, which is rich in G+C content, is smaller in size. This feature is different from other environmental members of this genus. Pathogenicity is directly related to the possession of a plasmid called pVAP (Giguere *et al*., 1999; Hondalus and Mosser, 1994; Takai *et al*., 1991). These plasmids are circular and harbour a PI that bears virulence genes. The sequence of this region is very different from the rest of the plasmid and is believed to be acquired through HGT (Letek *et al*., 2008).

The plasmid is prone to acquire genes through HGT as it belongs to CURV family and comprises, among others, a niche-adaptive variable region (VR) which is horizontally acquired and plays a vital role in niche adaptation. This is the reason that VR corresponds to PI in *R. equi* but in other species it executes different functions (Letek *et al*., 2010). Therefore, HGT is very significant in *R. equi* for acquiring characteristics. The horizontally acquired DNA in *R. equi* can account for upto a third of the plasmid DNA. Even plasmids themselves are highly mobilizable (Letek *et al*., 2010). Compared to the chromosome, the plasmids have greater proportion of DNA mobilization genes and niche-specific elements that enable them to adapt to a new environment successfully, for example the presence of PI for intracellular survival (Letek *et al*., 2008). Therefore, the plasmids of *R. equi* are not only vital for the plasticity of the genome but also for niche (host) adaptability (Letek *et al*., 2010).

It is hypothesized that it is HGT that conferred the saprophytic ancestor of *R. equi*, its preliminary capabilities to survive a new mode of life i.e. intracellular (Letek *et al*., 2008) (Figure 4.2). Initially in the environment free living protists like *Acanthamoeba* which are considered primitive macrophages (Vieira *et al*., 2015), might have provided training grounds for learning and improving the skills as a

pathogens through the co-option of core bacterial structures and functions. Later, through changes in gene regulation and functional modifications in proteins, they were able to infect animals by surviving in the macrophages. This leads to the hypothesis that **"**ability to survive in amebae may be pre-adaptive for existence of *R. equi* as pathogens in macrophages and thus horizontal acquisition of PI by *R. equi* ancestor (an original commensal) made it pathogenic by conferring the ability for intramacrophagal survival".

In order to trace this back, infections were setup using *Acanthamoeba* (MN-7) and *R. equi* plasmid-bearing (103^+) and plasmid-cured (103^-) strains. However, before starting *Acanthamoeba* infections, infections in macrophages were performed and validated to work out the survival dynamics of *R. equi* in macrophages under laboratory conditions with available strains of *R. equi*. Later these results were applied to compare the role of plasmid temperature in survival of *R. equi* in *Acanthamoeba*. Initially, murine macrophages J774A.1 and *R. equi* WT strains 103^+ and 103^- were used. Proliferation of only plasmid-bearing 103^+ *R. equi* was seen while the plasmid-cured 103^- strain could not sustain its survival in macrophages. The 103^+ strain showed some proliferation by 24h and the major boost was by 48h (Figure 4.9). Similar findings were also observed with another murine macrophage cell line RAW264.7 (Figure 4.10) which supported the initial observation in J774A.1 that the intracellular survival of *R. equi* in macrophages is plasmid dependent.

Different techniques of infection have been used to study the intracellular survival of bacterial pathogens in macrophages (Forest *et al*., 2010; Hondalus and Mosser, 1994; Luhrmann *et al*., 2004). The murine macrophage cell line J774A.1 used in the current study is commonly used to study intracellular survival of bacterial pathogens in macrophages (Hondalus and Mosser, 1994; Kuehnel *et al*., 2001; Valentin-Weigand *et al*., 1996). However, various types of cells have been used by different workers to conduct infection assays with *R. equi* with varying outcomes. These include murine J774E macrophages (Luhrmann *et al*., 2004; Pei *et al*., 2006; Sydor *et al*., 2008), murine macrophage-like cells (Toyooka *et al*., 2005), human monocyte cell line U937 (van der Geize *et al*., 2008), bone marrow derived macrophages (Darrah *et al*.,

2000), murine peritoneal macrophages, equine alveolar macrophages, J774A.1 murine monocyte-macrophage-like cells (Hondalus and Mosser, 1994).

The nitrite levels were also monitored during the infection assays for control as well as for infected cells which actually indicate the level of activation of macrophages. Any macrophages with unusually high levels of nitrite level indicate activation state which is not desirable as activated macrophages are strong killers of microbes (Nathan and Hibbs, 1991) and produce cellular reactive oxygen intermediates (ROIs) upon activation (Babior, 1984; Halliwell and Gutteridge, 1984) as well as reactive nitrogen intermediates (RNIs), which include nitric oxide (NO), nitrite ($NO2^-$) and nitrate ($NO3^-$) (Marletta *et al*., 1988). Therefore, it was important to make sure the macrophages were in ground state. The levels of nitrite for the control cells were fairly low indicating non-activated state. However, infected macrophages showed higher levels due to presence of pathogenic *R. equi* in the cells (Figure 4.9b and 4.10b).

To reinforce the findings, further infections were carried out using other *R. equi* WT strains including equine isolate RE1, bovine isolate 1571 and porcine isolate 1593. All of these strains also showed a similar proliferation trend as seen with the 103^+ strain (Figure 4.11). Further consolidation of intracellular survival assay for *R. equi* in macrophages was made by testing mutants with known effects of mutations. These included mutants with mutation in *arsB*, *choE*, *supAB*, *mce1*, *chori* and *ureA* genes (Figure 4.12).

The *arsB* gene is directly involved in the survival of *R. equi* in unfavourably high environmental arsenic levels and confers this pathogen ability for high arsenite-resistance (Cai *et al*., 2009). However, under the normal laboratory condition where the organism is not exposed to arsenic, this gene is no longer expressed. As expected, the mutant strain had no effect of mutation and showed proliferation in the murine macrophages. The *choE* is a cholesterol oxidase (exoenzyme) encoding gene of *R. equi* and it has long been considered as a potential virulence factor and was termed 'equi factor' (Prescott JF, 1982). It is secreted by both virulent and nonvirulent

strains of *R. equi* which damages host cell membranes in association with phospholipases (Hondalus, 1997). It is the major secretory protein of *R. equi* out of the total number of 48 proteins identified (Barbey *et al*., 2009). In the current study, *R. equi choE* mutant retained its ability to survive and grow inside the macrophages. This finding was in consistence with that of Pei *et al*., 2006 who showed that there was no effect of mutation in *choE* mutant as demonstrated by the macrophage, mice and foal infections.

The *supA* and *supB* are two adjacent genes which form part of a cluster involved in cholesterol catabolism are important for survival of *R. equi* on cholesterol but not in macrophages (van der Geize *et al*., 2008) which is demonstrated in the current study as well. The *R. equi mce1* mutant strain was unable to grow inside the macrophages at all indicating an effect of mutation and role of *mce1* gene in virulence of *R. equi*. The *mce1* is not a plasmid gene rather it is found on *R. equi* chromosome and is one of the three genes forming a cluster there (Rahman *et al*., 2003; Rengarajan *et al*., 2005). A homologous *mce1* gene is also found in closely related *Mycobacterium tuberculosis* where it plays a vital role in the intracellular survival of bacteria (Arruda *et al*., 1993; Rengarajan *et al*., 2005).

The *chori* gene encodes chorismate mutase, one of the important metabolic enzymes which catalyzes the initial steps in aromatic amino acid biosynthesis (Dosselaere and Vanderleyden, 2001). Chorismate mutase is important for intracellular survival and proliferation of *R. equi*. The deletion mutant was significantly deficient in growth at 48h compared to the WT strain. These findings were in agreement with those of Letek *et al*., (2010). The *ureA* encodes a urease which helps in release of ammonia and thereby enabling *R. equi* to survive the hostile acidic environment like the one in vacuoles in macrophages (Letek *et al*., 2010). Mutation had no effect on growth of this mutant.

Overall, the validation results, using different macrophage cell lines, different WT *R. equi* strains and mutants, all showed consistency of the initial infections using J774A.1. Therefore, the macrophage experiments concluded that *R. equi* could

survive the intracellular environment of macrophages well in a plasmid dependent manner. This was based on the observation that only the plasmid-bearing WT strains (103^+, RE1, 1571 and 1593) could survive in macrophages but the plasmid-cured strain (103^-) could not, indicating the potential role of the plasmid in macrophagal survival. A significantly higher proliferation of *R. equi* was seen by 48h without major killing of macrophages which is a desirable characteristic as the 48h period is fairly long enough to monitor the effect of intracellular survival. However, these findings were in contrary to the methods which ended up with massive killing of macrophages (Toyooka *et al*., 2005).

The method of infection was then applied to monitor the role of plasmid in intracellular survival of *R. equi* in *Acanthamoeba*. Initial observations indicated a much lower uptake of *R. equi* by *Acanthamoeba* (MN-7) than macrophages and this was not improved simply by increasing MOI (Figure 4.13), depriving *Acanthamoeba* of the nutrients (Figure 4.14) or lowering temperature (Figure 4.15).

For the infection assay to be used for studying the intracellular survival of *R. equi* in *Acanthamoeba* MN-7, modifications had to be made for *Acanthamoeba* because of some detachment of amoeba cells at 37°C (Figure 4.3). Therefore, at each time point the number of *Acanthamoeba* cells was counted and associated with the number of bacteria recovered from these cells to get a more reliable number of bacteria at each time point (Figure 4.4). The final graph in this case was thus plotted between time (h) and the ratio that was obtained by dividing the number of bacteria by the number of amoeba cells. Experiments at 37°C indicated a plasmid dependant survival of *R. equi* where plasmid-bearing strain (103^+) showed better survival than the plasmid-cured strain (103^-) especially at 24h, depicting the role of plasmid in survival of *R. equi* in *Acanthamoeba* as well (Figure 4.16). These findings were further strengthened by the inability of *R. equi* 103^+ to show proliferation/survival at 22°C (Figure 4.17), indicating the significance of *R. equi* virulence plasmid in survival in *Acanthamoeba* as well.

Although these results were conclusive, attempts were made to further consolidate the findings. Among these, the first attempt involved prevention of phagosomal acidification (by using ammium chloride pre-treatment for amoeba cells) and use of acid stress (by exposure of amoeba cells to low pH=5.0). Both these interventions, improved the internalization of *R. equi* by *Acanthamoeba* MN-7 cells at t=0 of infection, however, the results of complete infection assays showed a negative impact of these pre-treatments as manifested by the failure of *R. equi* to grow intracellularly in amoeba cells (Figures 4.18 and 4.19). This was probably because of unexpected more aggressive behaviours of treated *Acanthamoeba* cells towards *R. equi*. The effect of increased uptake of *Campylobacter jejuni* by lowering pH has also been observed by Axelsson-Olsson *et al*., 2010 although effect of long term survival was not investigated. Similarly, lysosomal acid neutralization has been used to increase the uptake of bacteria (Akya *et al*., 2009; Medina *et al*., 2014) with similar results, however, long term survival was not studied.

To further consolidate the findings of *R. equi* survival at 37°C, an alternate lower temperature was identified where amoebae do not suffer detachment from the surface of the 24-well plate while at the same time *R. equi* virulence genes are also expressed. For this purpose a titration curve was needed that shows expression state of virulence genes at regular intervals of temperature. Although it is known that *R. equi* virulence is not expressed below 30°C (Takai *et al*., 1996) the precise regulation of various virulence genes with increasing temperature upto 37°C has not been deduced. For this purpose, four PI genes pVAPA_*vapA*, pVAPA_*vapC*, pVAPA_*vapF* and pVAPA_0056 were selected and the relative expression of these genes was worked out at 37, 36, 34, 32 and 30°C compared to 22°C (Figure 4.20).

The data obtained indicated 34ºC as the possibly reasonable temperature for the infection assay with *Acanthamoeba* MN-7 and *R. equi*. However, to confirm that expression at 34°C was practically adequate for supporting the intracellular survival of *R. equi*, macrophages (J774.A1) infections with *R. equi* at 34ºC were performed. *R. equi* 103^{+} was still able to proliferate intracellularly but not 103^{-} (Figure 4.21-a), which confirmed the adequacy of this temperature to retain virulence *in vivo*. Similar

findings were observed for infections of *Acanthamoeba* at 34ºC where 103^+ strain was able to show better survival at 24h but not 103^- (Figure 4.22-a).

These results confirm the role of plasmid in the survival of both in macrophages as well in *Acanthamoeba*. Parallel infections were also conducted at 22ºC in both macrophages (actual 25ºC, the lowest temperature reachable by the incubator) (Figure 4.21-b) and *Acanthamoeba* (Figure 4.22-b) to see the effect at virulence deactivated temperature. In *Acanthamoeba*, as expected, no difference of survival between 103^+ and 103^- was observed. However, in case of macrophages the experiment results were inconclusive due to inability of J774.A1 macrophages to sustain their morphological and physiological integrity at this low temperature. As a result massive growth of 103^+ as well as 103^- was observed probably indicating a loss of function of macrophages. Overall, these findings confirm that like macrophages intracellular survival of *R. equi* in *Acanthamoeba* MN-7 is plasmid and temperature dependent.

Further experiments were conducted to confirm the upregulation of PI virulence genes of *R. equi* during infection of macrophages as well as *Acanthamoeba*. Intracellular gene expression for *R. equi* virulence genes *vapA*, *vapC* and *vapF* in infected macrophages and *Acanthamoeba* was determined at 24h of infection. In macrophages, *vapA* was the most expressed gene with >18 fold increase in expression followed by *vapC* with 18 fold increase and *vapF* with 5 fold increase (Figure 4.23-a). These findings are consistent with Ren and Prescott (2003). In case of *Acanthamoeba* infection, *vapA* with ~12 fold increase was the most expressed virulence gene followed by *vapC* with ~6 fold and *vapF* with ~3 fold increase (Figure 4.23-b). This clearly indicates that as far as *vapA*, *vapC* and *vapF* are concerned, the same virulence genes are expressed for the survival of *R. equi* in macrophages as well as *Acanthamoeba* MN-7. However, further investigation is required with more PI genes, possibly involved in virulence, to spot if any genes are differentially expressed in macrophages and *Acanthamoeba*.

4.5. Conclusion

The findings of this study indicate a clear role of *R. equi* plasmid (PI) as a shared element used for intracellular survival in both macrophages and *Acanthamoeba* (MN-7). Intra-amoebal survival is temperature dependent which further confirms role of plasmid in intracellular survival. Moreover, similar virulence genes of PI (*vapA*, *vapC* and *vapF*) are upregulated during infection of macrophages and *Acanthamoeba* (MN-7). These results seem to support the hypothesis that the ability to survive in amebae may be pre-adaptive for the existence of *R. equi* as pathogens in macrophages and thus horizontal acquisition of PI by *R. equi* ancestor (an original commensal) made it pathogenic by conferring the ability for intra-macrophagal survival. Therefore, it seems logical to believe that following the acquisition of virulence genes through HGT, the virulence trait selection role of *Acanthamoeba* played role in the emergence of pathogenic potential and in transformation of saprophytic environmental bacterium into pathogenic intracellular bacteria. The amoebae and macrophages have already been regarded as evolutionary distant amoeboid cells (Singer, 2010). At the same time this study also concludes that *Acanthamoeba* can act as an environmental host for plasmid-bearing *R. equi* because of their potential to survive the hostile environment within *Acanthamoeba*. This is concerning for an emerging human pathogen like *R. equi* and requires further investigation to widen the understanding of the impact amoebae may exert on human and animal health through their interaction with *R. equi*.

4.6. Further investigations

Although the current study provides a comprehensive view of the impact of *Acanthamoeba* on bacteria with the focus on *R. equi*, there are still different areas that can be explored further. A detailed study of the comparison of *R. equi* genes putatively involved in the survival of these bacteria in *Acanthamoeba* and macrophages can further highlight the level of similarity of intracellular survival mechanisms involved in both these types of cells that can further signify the pre-adaptive role of amoebae in selection of virulence traits in *R. equi*. More infections of *R. equi* using various environmental isolates of *Acanthamoeba* and even other protozoa can broaden the understanding of the role of these free living protozoa in serving as environmental reservoirs for *R. equi* and emergence of virulence traits of these bacterial pathogen.

Chapter 5

Overall Discussion and Future Perspectives

This study aimed at the exploration of the mutual impact of interaction between *Acanthamoeba* and bacteria on each other. Overall, the research is divided into two main areas *viz* the effect of bacteria on *Acanthamoeba*, and the effect of *Acanthamoeba* on bacteria as reservoirs; two examples of emerging human pathogens-*Arcobacter butzleri* and *Rhodococcus equi* were used. The overall significance of this study, in addition to exploring the influence of bacteria and *Acanthamoeba* on each other, was to pre-empt the eventual consequences of these interactions on human and health.

The impact of available bacteria as food source is influential on *Acanthamoeba* genotypes (Chapter 2). The wide use of only *Escherichia coli* as the monoxenic source of food for the isolation of *Acanthamoeba* from environmental and clinical samples (Chung *et al*., 1996; Ertabaklar *et al*., 2007; Kong *et al*., 1995; Lorenzo-Morales *et al*., 2005a; Rahdar *et al*., 2012; Reyes-Batlle *et al*., 2014; Rezaeian *et al*., 2008; Tsvetkova *et al*., 2004) has identified 19 reported T types of *Acanthamoeba* (Corsaro and Venditti, 2010; Gast *et al*., 1996; Hewett *et al*., 2003; Horn *et al*., 1999; Lanocha *et al*., 2009; Magnet *et al*., 2014; Nuprasert *et al*., 2010; Qvarnstrom *et al*., 2013; Stothard *et al*., 1998) and another (T20) also suggested recently (Fruest, 2015). However, an important question emerges that "if bacteria other than *E. coli* are used for isolation of *Acanthamoeba*, will it affect the recovery of genotypes so isolated?" If it is so, then there may be some genotypes of *Acanthamoeba* that go masked or do not successfully emerge during isolation due to possible preference of different genotypes for different types of bacteria?

This question has never been addressed in detail, by linking the types of bacteria and T types of *Acanthamoeba* recovered, although in some studies *E. coli, Pseudomonas aeruginosa*, *Klebsiella aerogenes*, *Klebsiella ozaenae* were found to be good food for amoebae while *Staphylococcus aureus* were difficult to digest and *Klebsiella ozaenae* resulted in unusual digestion pattern by amoebae (Pickup *et al*., 2007; Weekers *et al*., 1993). Now that the genotypic classification system of *Acanthamoeba* has been established with over 1800 18S rRNA gene sequences of *Acanthamoeba* deposited in the gene banks (Fuerst, 2014), this question could probably be better addressed by

using different types of bacteria for isolation of *Acanthamoeba* followed by detailed phylogenetic comparison with morphological characteristics, pathogenic characteristics and bacterial endosymbiotic profiles as additional comparative parameters. The same was followed in the current study using two different types of bacteria; the Gram+ve *Enterococcus* and the Gram–ve *Arcobacter* both of which are also found in the environment (Byappanahalli *et al*., 2012; Collado and Figueras, 2011) while *E. coli* was used as control for isolation of *Acanthamoeba* from soil samples.

However, before addressing the question directly, attention was paid to the optimization of *Acanthamoeba* isolation procedure from soil because of the wide variations of this procedure in terms of the amount of sample, control of fungal contamination, choice of growth media for axenic culture and overall lengthy time for isolation, despite the common philosophy i.e. use of bacteria for isolation of amoebae followed by axenization for routine culturing (Chung *et al*., 1996; Ertabaklar *et al*., 2007; Kong *et al*., 1995; Rahdar *et al*., 2012; Todd *et al*., 2014; Tsvetkova *et al*., 2004; Walochnik *et al*., 2000; Zanella *et al*., 2012). The optimized method (Figure 2.12) was not only simple but also helped overcome the limitations of isolation and compared to the traditional method (Maciver *et al*., 2013) significantly reduced the overall time of isolation (Table 2.4).

To study the effect of bacteria on *Acanthamoeba* in terms of diversity, a total of 102 soil samples were randomly collected from various parts of England and Scotland (Appendices IA, IB, IC) and processed separately on *E. coli*, *Enterococcus* and *Arcobacter*. The comparison of the three types of isolates of *Acanthamoeba* obtained by using *E. coli* (Eco), *Enterococcus* (Ent) and *Arcobacter* (Arc) uncovered that the three types of isolates were different in number of ways. The sequence analysis of ASA.S1 fragment of 18S rRNA of isolates revealed that the presence a particular type of bacteria could affect the genotypes specially the subgroups and subtypes of *Acanthamoeba* with the emergence of T2/6 isolates, more T4 subtypes and isolates having very divergent sequences (T13/16) not recovered with *E. coli*. The Arc isolates showed greater divergence of 18S rRNA sequences than Eco isolates, meaning that

the sequences of Arc and Ent isolates were not only different from the Eco isolates but also they had greater divergence from the already reported sequences.

The Eco isolates were different from Ent and Arc isolates in the T types recovered. The Eco isolates included T2(6%), T4(89.2%), T11(2.4%) and T13(2.4%); Ent isolates with the narrowest diversity of T types comprised of only T4(95.1%) and T16(3.7%) while 1.2% of isolates were intermediate T13/T16; Arc isolates, in contrary, consisted of T2(14.3%), T2/6(2.4%), T4(78.6%), T13(2.4%) and 2.4% sequences that were intermediate 13/T16. This indicates that by using *Arcobacter* more T2, T2/6 and T13/16 types can be isolated than with *E. coli* which is a significant difference while *E. coli* is more efficient in isolating T11 although they only constituted 2.4% of total Eco T types. One common thing among all the three isolate groups (Eco, Ent and Arc) was that the T type T4 comprised the major genotype of *Acanthamoeba.* T4 has been documented to constitute the major T type among the environmental isolates (Maciver *et al*., 2013).

In the current study, however, on further examination of T4 sequences, it was found that there were remarkable differences among the T4 subgroups (T4-A, -B, -C, D, E, F and N) and subtypes (T4-1 to T4-39). *E. coli* was most efficient only in isolating T4-A (54.1% vs 47.0% in Ent and 28.8% in Arc); *Enterococcus* was most efficient in isolating T4-C (11.1% vs 1.3% in Eco and none in Arc) and T4-D (11.1% vs 8.1% in Ent and none in Arc); while *Arcobacter* was most efficient in isolating T4-B (19.7% vs 16.2% in Eco and 7.4% in Ent), T4-E (34.8% vs 12.3% in Ent and 9.5% in Eco) and T4-N (16.7% vs 11.1% in Ent and 10.8% in Eco).

Differences were also pronounced among the T4 subtypes of the three isolate groups. Other than the common subtypes T4-1, T4-20 and T4-32 were unique to Eco isolates; T4-7 and T4-27 were unique to Ent isolates; while T4-13 and T4-34 were found to be unique to Arc isolates. This indicates differential preferences of various subtypes for different bacterial types (*E. coli, Enterococcus* and *Arcobacter*). Furthermore, while T4-36 emerged as the biggest T4 subtype in Eco and Ent isolates, in opposition T4-12 was the major subtype among Arc isolates. The isolate groups also had differences in

the cyst shapes; Eco isolates were mostly star-shaped as compared to round shaped cysts in Ent and Arc isolates.

The pathogenic characteristics (thermotolerance and osmotolerance), however, were not quite useful in differentiating the three isolate groups as no effect of osmotolerance or thermotolerance at 41°C could be observed which might be due to the environmental nature of the samples (Landell *et al*., 2013; Lorenzo-Morales *et al*., 2005b; Niyyati *et al*., 2013).

Inability to recover all the genotypes of *Acanthamoeba* may cause misinterpretation actual prevalence of BEs. This was manifested by the final comparative parameter i.e. bacterial endosymbiotic profile of the *Acanthamoeba* isolates. The three isolate groups harboured different percentages and types of BEs they carried inside with Eco isolates having the lowest percentage (7.8%) while Arc isolates had the highest (15.7%) and Ent isolates had intermediate percentage of BEs (12.9%). This indicates greater association of BEs with Arc isolates than Eco isolates. Furthermore, it was found that although T4 was the only Type in Eco and Ent isolates to show presence of BEs, Arc isolates also had BEs in T2 T types as well. Similarly, the potential of different T4 subgroups and subtypes to harbour BEs was also different. Among Eco isolates only T4-B and T4-N had BEs compared to T4-A, T4-B, T4-E and T4-N subgroups. Similarly, on T4 subtypes level, Eco isolates had BEs in T4-23 and T4-36 only compared to T4-12, T4-23, T4-35 and "others" group of Arc isolates. Furthermore, only *Candidatus* Procabacter was detected in Eco isolates compared to *Candidatus* Procabacter, *Massilia* sp. and uncultured bacterial types in Arc isolates. No previous direct study has been made to compare the association of BEs with the *Acanthamoeba* subgroups or subtypes.

The results of this study imply that the *Acanthamoeba* genotypes may go masked due to use of a particular type of bacteria in laboratory isolation conditions. This, in turn, can also mask the BEs they harbour. Overall, this may lead to misinterpretation of prevalence of T types, subgroups, subtypes and any other intermediate sequences together with the BEs. A realistic understanding of the prevalence of various genotypes

of *Acanthamoeba* is essential for designing and implementing control and treatment strategies. Although amoebae serve as the major predators of bacteria in soil and thus regulate the bacterial population (Schuster *et al*., 1993), it is interesting that their own diversity, in turn, is also dependent upon the population of a particular type of bacteria in the environment which may favour selective outnumbering of certain genotypes.

The impact of *Acanthamoeba* on different bacterial pathogens has been studied such as *Legionella pneumophila* (Rowbotham, 1980), *Mycobacterium* sp. (Adekambi *et al*., 2006; Cirillo *et al*., 1997) and *Vibrio cholera* (Abd *et al*., 2010), however, the various aspects of this important relationship have not been investigated in detail in two emerging human pathogens *viz A. butzleri* and *R. equi*, the area which was explored as part of the second main area of the current study i.e. impact of *Acanthamoeba* on bacteria.

Although the significance of protozoans in the environment has long been realized specially the importance of predatory protozoans in regulating bacterial populations (Curds and Fey, 1969; Habte and Alexander, 1977; Singh, 1941; Stout, 1973), however, the dangerous role protozoans play as reservoirs and training grounds for human pathogens has only recently become the focus of academic investigation (Amann *et al*., 1997; Barker and Brown, 1994; Fields, 1996; Ly and Muller, 1990). This revolutionary finding gave rise to the concept that protozoans like *Acanthamoeba* play a vital role in the evolution of pathogenic environmental bacteria and, therefore, protists are the "missing link" between ecology and pathology (Barker and Brown, 1994; King *et al*., 1988; Ly and Muller, 1990). To further explore this, the interaction of two emerging human pathogens, *A. butzleri* (Chapter 3) and *R. equi* (Chapter 4), with *Acanthamoeba* was investigated to understand the role of amoebae in acting as the missing links as well as the possible threat posed by amoebae in modulating the pathogenic traits of these pathogens which may give rise to any serious consequences for human health.

A. butzleri were found to be modulated as a result of interaction with *Acanthamoeba*. A number of important observations were made in this study which, taken together,

suggest a potential role of *Acanthamoeba* in acting as an environmental reservoir for *A. butzleri.* These bacteria were found to be easily located by *Acanthamoeba* because of the strong chemotactic attraction by the latter (Figure 3.66-d). Moreover, they had great potential to adhere onto the surface of the amoeba cells in the form of a cap and the motile bacteria could be seen beating inside the vacuoles within a few minutes of exposure to *Acanthamoeba* (Figure 3.15).

The bacteria were also found to exploit the similar mechanisms as usually used by the pathogens; being taken up by the amoeba cells, although no evidence of coiling phagocytosis was seen as in *Burkholderia pseudomallei* (Inglis *et al*., 2000) and *Legionella pneumophila* (Bozue and Johnson, 1996). Entry into the cell appeared to depend upon monosaccharide sugar receptors on the cell surface of amoeba as reported for other bacteria as well (Akya *et al*., 2009). Phagocytosis was a complex process which required various cellular signalling pathways including actin polymerization, PI3K, PTPs and vATPases. Survival in amoeba was found to be related to the requirement for defying the acidification processes directly as well as indirectly such as inhibiting lysosomal enzymes, preventing lysosome-phagosome fusion, avoiding phagosome acidification and impacting on the intracellular transport system (section 3.3.3.4. and 3.3.3.5.).

A. butzleri were found to exploit amoebae as environmental reservoirs, not only for survival but also to improve their pathogenic potential as manifested by their ability for limited proliferation/lysis (Figures 3.30 and 3.33) and long term intracellular survival for upto two weeks normally or even upto 42 days as a results of enhanced pathogenic potential conferred by the repeated passages through amoeba cells (Figure 3.38) which strengthens the role of amoebae in the selection of pathogenic traits of bacteria. This phenomenon has also been observed in *L. pneumophila* (Cirillo *et al*., 1994). The proliferation and rupturing of *Acanthamoeba* cells by *A. butzleri* (although limited) can ensure transportation of these bacteria in water sources to wide areas. Intra-amoebal survival of bacteria is hypothesized as an important step in their training for adaptation to mammalian cells (Molmeret *et al*., 2005).

A. butzleri under altered environmental conditions (presence of nicotinic acid) could change their pathogenic traits towards *Acanthamoeba* and better exploited amoebae as biological reservoirs with better survival (section 3.3.7.). *Acanthamoeba* also supported extracellular survival (but not excystment) of *A. butzleri* by releasing growth supporting factors although they were not paid back by *A. butzleri* in the same way (section 3.3.8.).

In summary, based upon these interaction studies of *A. butzleri* and *Acanthamoeba*, it is possible that *Acanthamoeba* facilitate *A. butzleri* in a number of ways and are thus potential environmental reservoirs and breeding sites for these emerging human pathogens. This is concerning because ignoring the role of *Acanthamoeba* as the reservoirs and as pathogenicity-trait-modulators of *A. butzleri*, can not only result in the risk of outbreaks of these bacteria but also the treatment and control strategies may be compromised.

A similar kind of interaction of *Acanthamoeba* was observed with the other emerging human bacterial pathogen *R. equi*, the Gram+ve actinomycete rhodococci closely resembling *Mycobacterium* sp. and once thought to infect horses only (Cohen *et al*., 2015; Puthucheary *et al*., 2006). The acquisition of virulence genes through HGT on PI of this bacterium is believed to have conferred this originally saprophytic bacterium with pathogenic capabilities. The co-existence of free-living protozoa like *Acanthamoeba* might have provided training grounds for *R. equi* in their adaptation towards survival in macrophages, thereby paving way for a new era of pathogenic mode of life for *R. equi*.

This observation gives rise to the hypothesis that "ability to survive in amebae may be pre-adaptive for *R. equi* existence as pathogens in macrophages and thus horizontal acquisition of PI by *R. equi* ancestor made this originally commensal ancestor pathogenic by conferring the ability for intra-macrophagal survival". This hypothesis can be trialled on the basis of tracking back the survival of *R. equi* in *Acanthamoeba* in a plasmid-dependent manner and commonalities of intracellular survival strategies.

To test the hypothesis, the intra-macrophagal survival pattern of *R. equi* was first established for comparison with intracellular survival in *Acanthamoeba*. This involved the consolidation of an assay using two murine macrophage cell lines (J774A.1 and RAW264.7) and a number of different wildtype and mutant strains. The use of wildtype (WT) plasmid-bearing (103^+) and plasmid-cured (103^-) strains indicated a plasmid-dependent proliferation pattern of bacteria that involved slight increase in number of bacteria by 24h followed by a major increase by 48h, There was no survival of plasmid-cured 103^- strain (section 4.3.3.).

Before applying the method to study survival of *R. equi* in *Acanthamoeba* (MN-7), some modifications were made to compensate for the detaching host cells from the 24-well plate at 37°C. This involved counting of amoebal cells at each time point in addition to the bacterial CFUs and using the ratio (No. of bacteria/No. of amoeba cells) against time to monitor the intracellular survival of *R. equi* in *Acanthamoeba* MN-7. *Acanthamoeba* cells were then infected with *R. equi* WT strains 103^+ and 103^-. *R. equi* also showed a plasmid-dependent and temperature-dependent survival in amoebal cells (section 4.3.4.2.). The basic survival pattern of *R. equi* in *Acanthamoeba* was comparable to that in macrophages, showing proliferation by 24h of infection, however, compared to macrophages the rate of internalization was lower in *Acanthamoeba* and there was no further proliferation by 48h.

These findings were further consolidated by carrying out infections at a lower temperature to prevent detachment of amoebal cells. The profile for relative expression of *R. equi* plasmid virulence genes (*vapA*, *vapC*, *vapF*, 0056) was first completed at various temperatures. All of these genes except 0056 were found to be strongly regulated by temperature and 34°C seemed to be a good temperature corresponding to no significant detachment of amoebal cells. However, to confirm the adequacy of this temperature to support intracellular survival of *R. equi*, infections were first made in macrophages at this temperature which showed a proliferation similar to that at 37°C. Infections in *Acanthamoeba* MN-7 at 34°C showed a similar survival pattern as at 37°C which was plasmid- and temperature-dependent (section 4.3.4.4.2.). To further compare the intracellular survival strategies used *R. equi* for survival in macrophages

and *Acanthamoeba*, intra-macrophagal and intra-amoebal expression of virulence genes (*vapA*, *vapC* and *vapF*) was investigated. The *vapA* and *vapC* were found to be strongly expressed followed by *vapF* in both the cell types (Figure 4.23) indicating shared intracellular survival strategies used by *R. equi* in macrophages as *Acanthamoeba*. Similar findings were first reported in case of *L. pneumophila* (Cianciotto and Fields, 1992).

These results indicate that although *R. equi* show limited survival potential in amoeba cells as compared to macrophages yet they share the basic strategies which seems to support the hypothesis that adaptation of bacteria to amoebae served as a preliminary step in the adaptation to an intra-macrophagal environment (Molmeret *et al*., 2005). Furthermore, the ability of amoebae to support the growth of plasmid-bearing *R. equi* indicates their role in acting as environmental reservoirs which can host pathogens of a threat for human and animal health.

Acanthamoeba may provide protection to *R. equi* and also may act as "Trojan horse" (Bozue and Johnson, 1996) by inserting them into human host as it has been observed for closely related *M. avium* (Cirillo *et al*., 1997).

Conclusion and future directions

It can be concluded from this study on the interaction between *Acanthamoeba* and bacteria that both these organisms are affected by each other. The originally conceived predator-prey relation is indeed far more diverse and complex than it apparently seems. A single source of bacterium cannot efficiently recover all genotypes present within an environmental sample, which may affect the outcomes of any prevalence or diagnostic studies of *Acanthamoeba* and ultimately treatment. While the diversity of various genotypes of *Acanthamoeba* is related to the presence of bacterial types, at the same time the fate of bacteria is also determined and modulated by amoebae as demonstrated by the intracellular survival capabilities of two emerging human pathogens-*A. butzleri* and *R. equi*.

In the case of *A. butzleri* it seems logical to believe that the virulence traits highlighted by amoebae as a result of intra-amoebal survival are actually present within the genome of the bacteria and are only "selected" by the amoebae (Greub and Raoult, 2004). Various factors appear to have played role in the transformation of the ancestral non-pathogenic environmental saprophytic form of *R. equi* to the pathogenic form having potential to infect humans; these include horizontal acquisition of virulence genes (eased by its genome plasticity properties), together with intra-amoebal virulence selection and co-option of core bacterial structures and functions. *Acanthamoeba* acted as environmental reservoirs for these bacteria not only to provide shelter and protection but also selecting natural pathogenicity under the survival pressure of their hostile intracellular environments. Therefore, the pre-adaptation of bacteria in amoebae opens doors for the new pathogens to inhabit the new host environment (humans or animals). Amoebae are, therefore, the strong candidates as the "missing links" between the ecology and the pathology of these pathogens. Such interactions not only explain the evolution of emerging bacterial pathogens but also warn of possible future threats, thus demanding a deeper insight into these interactions.

This study highlights the important aspects of *Acanthamoeba*-bacteria interactions, however, further investigations can help better understand the dynamics and versatility of this relation and the impact on human and animal health. The role of bacteria in

exposing the unsurfaced genotypes needs more extensive exploration by using a wide range of different types of bacteria to find out the significance of various bacteria in recovering *Acanthamoeba* from environmental or clinical samples and thus standardizing the possible combination of different bacterial types that can be used effectively to help isolate complete set of all the genotypes present in a sample. These measures can better help identify the prevalence of *Acanthamoeba* genotypes in the environment which is crucial for designing any control strategies for emerging bacterial pathogens through control of free living amoebae in the environment.

Moreover, in addition to investigating the similarities between the intracellular survival strategies in amoeba and natural host cells at the molecular and genetic level, it would be greatly helpful to explore the effect of amoebae on bacteria by studying the interactions of *A. butzleri* and *R. equi* with not only other environmental *Acanthamoeba* isolates but also with other free living protozoa including *Chilomonas* (flagellate) and *Tetrahymena* (ciliate).

Health risks associated with the improved survival and replication capabilities of bacteria in free-living amoebae call for re-standardization of microbiological quality standards such as food-processing and drinking water treatments in addition to improvement of the sanitation protocols for better control of free-living protozoa and the pathogenic bacteria they harbour. In other words, a better understanding of the ways that bacteria and free-living protozoa interact with each other, and how such interactions can affect human health, will contribute towards designing and implementing better control and eradication strategies.

In conclusion, it is becoming more and more evident that the interaction between the amoebae and the bacteria, that was originally believed to be restricted to mere predation of bacteria (with eventual digestion by the former) extends up to the other extreme where bacteria multiply intracellularly and eventually kill amoebae. This relation is emerging to fall between these two ends at various levels. Both bacteria as

well as amoebae seem to impact each other as a consequence of their interaction. The more the interactions between the amoebae and bacteria are investigated, the more the width and the breadth of the two extremes of the amoeba-bacteria interactions will be unfolded. This will not only help in understanding of the diversity of this relation but also it will help in better designing and implementing control strategies for both the bacterial and amoebal infections.

Chapter 6

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Appendices

Appendix-IA

Details of the soil samples processed on *E. coli* for the isolation of *Acanthamoeba*

Soil samples		Comparison with reference sequences					Comparison with Genbank sequences			
Code	**Location**	**Max identity**	**T type**	**T4 subgroup**	**T4 subtype**		**Max identity**	**Coverage**	**T type**	**Genbank Accession No.**
					Nearest match	**Identity (%)**				
Eco-B01	Burnham	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Eco-B02	Burnham	99	T4	A	T4-16	100	99	100	T4	HM036180.1
Eco-B03	Burnham	100	T4	B	T4-16	96	100	100	T4	JQ678630.1
Eco-B04	Burnham	-	Not recovered	-	-	-	-	-	-	-
Eco-B05	Burnham	-	Contaminated	-	-	-	-	-	-	-
Eco-B06	Burnham	99	T4	A	T4-35	100	100	100	T4	EU146070.1
Eco-B07	Burnham	-	Not recovered	-	-	-	-	-	-	-
Eco-B08	Burnham	100	T4	A	T4-8	100	99	100	T4	EU273825.1
Eco-B12	Burnham	99	T4	A	T4-31	98	99	100	T4	EU273826.1
Eco-B13	Burnham	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Eco-E01	Edinburgh	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Eco-E02	Edinburgh	98	T4	D	Multiple?	92	99	100	T4	JQ678625.1|
Eco-E03	Edinburgh	100	T4	A	T4-36	98	100	99	T4	FJ807651.1
Eco-E04	Edinburgh	100	T4	A	T4-16	96	100	100	T4	GU936484.1
Eco-E05	Edinburgh	99	T4	A	T4-31	98	99	99	T4	KF318462.1
Eco-E06	Edinburgh	100	T4	N	T4-36	96	100	100	T4	KF928946.1
Eco-E07	Edinburgh	99	T4	E	T4-12	96	99	99	T4	KF928953.1
Eco-E08	Edinburgh	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Eco-E09	Edinburgh	99	T4	B	T4-23	100	100	100	T4	KF928942.1
Eco-E10	Edinburgh	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Eco-E11	Edinburgh	100	T4	A	T4-36	96	100	100	T4	JX494783.1
Eco-E12	Edinburgh	100	T4	A	T4-36	96	100	100	T4	JX494783.1
Eco-E13	Edinburgh	99	T4	B	T4-23	100	100	100	T4	KF928942.1

Eco-E14	Edinburgh	100	T4	A	T4-36	98	100	100	T4	JX423603.1
Eco-E15	Edinburgh	99	T4	E	T4-12	96	99	99	T4	KF928953.1
Eco-E16	Edinburgh	100	T13	-	-	-	100	100	T13	KF928948.1
Eco-E17	Edinburgh	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Eco-E18	Edinburgh	99	T11	-	-	-	100	100	T11	KJ801938.1
Eco-E19	Edinburgh	-	Contaminated	-	-	-	-	-	-	-
Eco-E20	Edinburgh	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Eco-E21	Edinburgh	100	T4	B	T4-23	100	100	100	T4	KF928942.1
Eco-M01	Manchester	98	T4	D	T4-26	100	100	98	T4	DQ264391.1
Eco-M02	Manchester	99	T4	B	T4-23	100	99	100	T4	KF928942.1
Eco-M03	Manchester	99	T4	C	T4-20	100	99	100	T4	JX441873.1
Eco-M04	Manchester	100	T4	A	T4-1	98	99	99	T4	KJ094663.1
Eco-M05	Manchester	99	T4	E	T4-12	100	100	100	T4	HF930501.1
Eco-M06	Manchester	100	T4	A	T4-1	100	99	99	T4	KC669509.1
Eco-M07	Manchester	100	T4	A	T4-31	100	99	100	T4	KF318462.1s
Eco-M08	Manchester	99	T4	B	T4-23	98	99	99	T4	KF928942.1
Eco-M09	Manchester	-	Contaminated	-	-	-	-	-	-	-
Eco-M10	Manchester	-	Contaminated	-	-	-	-	-	-	-
Eco-NB01	N Berwick	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Eco-NB02	N Berwick	100	T4	N	T4-?	88	100	100	T4	JX494771.1
Eco-NB03	N Berwick	98	T4	A	T4-8	100	99	100	T4	HF930502.1
Eco-NB04	N Berwick	100	T4	N	T4-36	96	100	100	T4	KJ094691.1
Eco-NB05	N Berwick	100	T4	A	T4-8	100	100	100	T4	EU168069.2
Eco-NB06	N Berwick	99	T4	A	T4-31	96	100	99	T4	JX494783.1
Eco-NB07	N Berwick	98	T4	B	T4-23	96	98	100	T4	KF928942.1
Eco-NC01	Newcastle	-	Contaminated	-	-	-	-	-	-	-
Eco-NC02	Newcastle	-	Contaminated	-	-	-	-	-	-	-
Eco-NC03	Newcastle	-	Contaminated	-	-	-	-	-	-	-
Eco-NC04	Newcastle	-	Not recovered	-	-	-	-	-	-	-
Eco-NC05	Newcastle	-	Not recovered	-	-	-	-	-	-	-
Eco-NC06	Newcastle	-	Not recovered	-	-	-	-	-	-	-

Eco-NC07	Newcastle	100	T4	B	T4-16	96	99	99	T4	JQ678630.1
Eco-NC08	Newcastle	98	T4	D	T4-36	96	99	100	T4	KF881880.1
Eco-NC09	Newcastle	98	T4	N	T4-?	88	99	100	T4	JQ678631.1
Eco-P01	Paddington	-	Not recovered	-	-	-	-	-	-	-
Eco-P02	Paddington	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Eco-P03	Paddington	99	T4	A	T4-16	100	99	100	T4	HM036180.1
Eco-P04	Paddington	99	T2	-	-	-	99	98	T2	KF928936.1
Eco-P05	Paddington	100	T4	B	T4-16	96	100	100	T4	JQ678630.1
Eco-P06	Paddington	99	T4	A	T4-35	100	100	100	T4	EU146070.1
Eco-P07	Paddington	98	T4	A	T4-8	100	99	100	T4	EU273825.1
Eco-P08	Paddington	-	Not recovered	-	-	-	-	-	-	-
Eco-P09	Paddington	100	T13	-	-	-	100	100	T13	KF928948.1
Eco-P11	Paddington	99	T4	E	T4-32	98	100	99	T4	HG797020.1
Eco-S01	Slough	99	T4	A	T4-31	98	99	100	T4	EU273826.1
Eco-S02	Slough	100	T4	B	T4-16	96	99	99	T4	JQ678630.1
Eco-S03	Slough	100	T4	N	T4-?	88	99	100	T4	JQ678631.1
Eco-S04	Slough	-	Not recovered	-	-	-	-	-	-	-
Eco-S05	Slough	99	T2	-	-	-	99	100	T2	AF019050.1
Eco-S06	Slough	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Eco-S07	Slough	100	T2	-	-	-	100	100	T2	U07411.1
Eco-SK01	S Kensington	100	T4	D	T4-?	82	99	100	T4	JQ678625.1
Eco-SK02	S Kensington	99	T4	E	T4-12	100	100	100	T4	FJ422512.1
Eco-SK03	S Kensington	100	T4	N	T4-36	96	100	100	T4	KF928946.1
Eco-SK04	S Kensington	-	Not recovered	-	-	-	-	-	-	-
Eco-SK05	S Kensington	99	T4	A	T4-31	98	99	99	T4	KF318462.1
Eco-SK06	S Kensington	100	T4	A	T4-36	96	100	100	T4	JX494783.1
Eco-SK07	S Kensington	99	T2	-	-	-	99	98	T2	KF928936.1
Eco-SK08	S Kensington	100	T4	A	T4-16	96	100	100	T4	GU936484.1
Eco-T01	Tilehurst	100	T4	N	T4-?	88	100	100	T4	JX494771.1
Eco-T02	Tilehurst	100	T2	-	-	-	100	100	T2	U07411.1
Eco-T03	Tilehurst	100	T4	A	T4-36	98	100	99	T4	FJ807651.1

Eco-T04	Tilehurst	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Eco-T05	Tilehurst	100	T4	B	T4-23	100	100	100	T4	KF928942.1
Eco-T06	Tilehurst	99	T4	E	T4-32	98	100	99	T4	HG797020.1
Eco-T07	Tilehurst	-	Not recovered	-	-	-	-	-	-	-
Eco-T08	Tilehurst	100	T4	A	T4-8	100	99	100	T4	HF930502.1
Eco-V01	Victoria	-	Not recovered	-	-	-	-	-	-	-
Eco-V02	Victoria	100	T4	N	T4-36	96	100	100	T4	KJ094691.1
Eco-V03	Victoria	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Eco-V04	Victoria	99	T4	D	T4-36	96	99	100	T4	KF881880.1
Eco-V05	Victoria	-	Not recovered	-	-	-	-	-	-	-
Eco-V06	Victoria	99	T4	A	T4-31	96	100	99	T4	JX494783.1
Eco-V07	Victoria	98	T4	B	T4-23	96	98	100	T4	KF928942.1
Eco-V08	Victoria	98	T4	D	T4-?	82	99	100	T4	JQ678625.1
Eco-V09	Victoria	99	T4	E	T4-12	100	100	100	T4	FJ422512.1
Eco-V10	Victoria	99	T11	-	-	-	100	100	T11	KJ801938.1
Eco-V11	Victoria	100	T4	A	T4-8	100	100	100	T4	EU168069.2
Eco-V13	Victoria	100	T4	A	T4-36	98	100	100	T4	JX423603.1

*"Others" subtypes did not have close resemblance with any of the reference T4 subtypes.

Appendix-IB

Details of the soil samples processed on *Enterococcus* for the isolation of *Acanthamoeba*

Soil Samples		Comparison with reference sequences					Comparison with Genbank sequences			
Code	Location	Max similarity	T type	T4 subgroup	T4 subtype		Max similarity	Coverage	T type	Genbank Accession No.
					Nearest match	Similarity (%)				
Ent-B01	Burnham	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Ent-B02	Burnham	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Ent-B03	Burnham	99	T4	N	Others*	93	99	100	T4	AM408796.1
Ent-B04	Burnham	-	Not recovered	-	-	-	-	-	-	-
Ent-B05	Burnham	-	Contaminated	-	-	-	-	-	-	-
Ent-B06	Burnham	98	T4	N	Others*	93	99	99	T4	KJ476515.1|
Ent-B07	Burnham	-	Not recovered	-	-	-	-	-	-	-
Ent-B08	Burnham	99	T4	C	T4-26	100	99	100	T4	DQ264391.1
Ent-B12	Burnham	96	T4	C	T4-26	100	100	100	T4	DQ087288.1
Ent-B13	Burnham	99	T4	C	T4-26	98	99	100	T4	AM412762.1
Ent-E01	Edinburgh	99	T4	A	T4-22	100	99	100	T4	HF930508.1
Ent-E02	Edinburgh	96	T4	C	T4-26	100	100	100	T4	DQ087288.1
Ent-E03	Edinburgh	99	T4	N	T4-36?	93	99	100	T4	AM408796.1
Ent-E04	Edinburgh	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Ent-E05	Edinburgh	100	T4	A	T4-31	100	100	100	T4	KF318462.1
Ent-E06	Edinburgh	99	T4	N	Others*	93	99	100	T4	AM408796.1
Ent-E07	Edinburgh	99	T4	E	T4-12	100	100	100	T4	FJ422512.1
Ent-E08	Edinburgh	98	T4	A	T4-8	100	99	100	T4	JX423609.2
Ent-E09	Edinburgh	100	T16	-	-	-	100	100	T16	AY026245.1
Ent-E10	Edinburgh	99	T4	A	T4-16	100	99	100	T4	HM036180.1
Ent-E11	Edinburgh	99	T4	E	T4-12	96	99	99	T4	KF928953.1
Ent-E12	Edinburgh	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Ent-E13	Edinburgh	99	T4	D	T4-26	98	100	100	T4	KJ094676.1

Ent-E14	Edinburgh	100	T4	A	T4-31	100	100	100	T4	KF318462.1
Ent-E15	Edinburgh	100	T4	A	T4-31	100	99	100	T4	KF318462.1
Ent-E16	Edinburgh	98	T4	E	T4-12	98	99	100	T4	JQ669660.2
Ent-E17	Edinburgh	99	T4	A	T4-35	100	99	100	T4	AY703004.1
Ent-E18	Edinburgh	100	T4	E	T4-12	98	100	100	T4	KF928953.1
Ent-E19	Edinburgh	100	T4	A	T4-31	100	100	100	T4	KF318462.1
Ent-E20	Edinburgh	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Ent-E21	Edinburgh	99	T4	C	T4-26	100	99	100	T4	DQ264391.1
Ent-M01	Manchester	99	T4	D	T4-26	100	100	100	T4	DQ264391.1
Ent-M02	Manchester	99	T4	B	T4-23	100	100	100	T4	KF928942.1
Ent-M03	Manchester	99	T4	C	T4-26	100	99	100	T4	DQ264391.1
Ent-M04	Manchester	100	T4	D	T4-27	100	100	100	T4	AY351644.1
Ent-M05	Manchester	99	T4	E	T4-12	100	100	100	T4	FJ422512.1
Ent-M06	Manchester	98	T4	A	T4-8	100	99	100	T4	JX423609.2
Ent-M07	Manchester	100	T4	A	T4-31	100	100	100	T4	KF318462.1
Ent-M08	Manchester	98	T4	E	T4-12	98	99	100	T4	JQ669660.2
Ent-M09	Manchester	-	Contaminated	-	-	-	-	-	-	-
Ent-M10	Manchester	-	Contaminated	-	-	-	-	-	-	-
Ent-NB01	North Berwick	99	T4	B	T4-23	100	100	100	T4	KF928942.1
Ent-NB02	North Berwick	99	T4	D	T4-26	98	100	100	T4	KJ094676.1
Ent-NB03	North Berwick	99	T4	D	T4-26	100	100	100	T4	DQ264391.1
Ent-NB04	North Berwick	99	T4	N	T4-36	95	99	100	T4	KJ476515.1
Ent-NB05	North Berwick	99	T4	A	T4-36	98	100	100	T4	KF318462.1
Ent-NB06	North Berwick	99	T4	C	T4-26	98	99	100	T4	AM412762.1
Ent-NB07	North Berwick	98	T4	B	T4-23	100	98	99	T4	KJ094684.1
Ent-NC01	Newcastle	99	T4	A	T4-22	98	100	100	T4	DQ087302.1
Ent-NC02	Newcastle	100	T4	A	T4-31	100	100	100	T4	KF318462.1
Ent-NC03	Newcastle	100	T4	A	T4-31	100	100	100	T4	KF318462.1
Ent-NC04	Newcastle	-	Not recovered	-	-	-	-	-	-	-
Ent-NC05	Newcastle	-	Not recovered	-	-	-	-	-	-	-
Ent-NC06	Newcastle	-	Not recovered	-	-	-	-	-	-	-

Ent-NC07	Newcastle	98	T4	B	T4-23	100	98	99	T4	KJ094684.1
Ent-NC08	Newcastle	100	T4	D	T4-27	100	100	100	T4	AY351644.1
Ent-NC09	Newcastle	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Ent-P01	Paddington	-	Not recovered	-	-	-	-	-	-	-
Ent-P02	Paddington	99	T4	A	T4-22	98	100	100	T4	DQ087302.1
Ent-P03	Paddington	98	T4	A	T4-8	100	99	100	T4	JX423609.2
Ent-P04	Paddington	99	T4	E	T4-12	100	100	100	T4	FJ422512.1
Ent-P05	Paddington	100	T16	-	-	-	100	100	T16	AY026245.1
Ent-P06	Paddington	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Ent-P07	Paddington	100	T4	D	T4-27	100	100	100	T4	AY351644.1
Ent-P08	Paddington	-	Not recovered	-	-	-	-	-	-	-
Ent-P09	Paddington	97	T13/16	-	-	-	99 (2/3)	100	T13	KF928948.1
Ent-P11	Paddington	100	T4	E	T4-12	98	100	100	T4	KF928953.1
Ent-S01	Slough	100	T4	E	T4-12	98	100	100	T4	KF928953.1
Ent-S02	Slough	99	T4	E	T4-12	100	100	100	T4	FJ422512.1
Ent-S03	Slough	98	T4	A	T4-8	100	99	100	T4	JX423609.2
Ent-S04	Slough	-	Not recovered	-	-	-	-	-	-	-
Ent-S05	Slough	100	T4	A	T4-31	100	100	100	T4	KF318462.1
Ent-S06	Slough	99	T4	A	T4-22	98	100	100	T4	DQ087302.1
Ent-S07	Slough	100	T4	A	T4-31	100	100	100	T4	T4
Ent-SK01	S Kensington	99	T4	C	T4-26	98	99	100	T4	AM412762.1
Ent-SK02	S Kensington	100	T4	A	T4-16	96	100	100	T4	GU936484.1
Ent-SK03	S Kensington	-	Contaminated	-	-	-	-	-	-	-
Ent-SK04	S Kensington	-	Not recovered	-	-	-	-	-	-	-
Ent-SK05	S Kensington	100	T16	-	-	-	100	100	T16	AY026245.1
Ent-SK06	S Kensington	99	T4	A	T4-7	98	99	100	T4	EU146070.1
Ent-SK07	S Kensington	99	T4	A	T4-31	98	99	99	T4	KF318462.1
Ent-SK08	S Kensington	99	T4	N	T4-36	95	99	100	T4	JX494774.1
Ent-T01	Tilehurst	99	T4	D	T4-26	100	100	100	T4	DQ264391.1
Ent-T02	Tilehurst	100	T4	D	T4-27	100	100	100	T4	AY351644.1
Ent-T03	Tilehurst	99	T4	A	T4-16	100	99	100	T4	HM036180.1

Ent-T04	Tilehurst	98	T4	A	T4-8	100	99	100	T4	EU273825.1
Ent-T05	Tilehurst	99	T4	B	T4-23	100	100	100	T4	KF928942.1
Ent-T06	Tilehurst	99	T4	N	T4-36	95	99	100	T4	KJ476515.1
Ent-T07	Tilehurst	-	Not recovered	-	-	-	-	-	-	-
Ent-T08	Tilehurst	99	T4	A	T4-36	98	100	100	T4	KF318462.1
Ent-V01	Victoria	-	Not recovered	-	-	-	-	-	-	-
Ent-V02	Victoria	98	T4	N	Others*	93	99	99	T4	KJ476515.1|
Ent-V03	Victoria	98	T4	C	T4-26	100	100	100	T4	DQ087288.1
Ent-V04	Victoria	99	T4	A	T4-35	100	100	100	T4	EU146070.1
Ent-V05	Victoria	-	Not recovered	-	-	-	-	-	-	-
Ent-V06	Victoria	98	T4	B	T4-23	100	98	99	T4	KJ094684.1
Ent-V07	Victoria	99	T4	A	T4-16	100	99	100	T4	HM036180.1
Ent-V08	Victoria	99	T4	A	T4-36	98	100	100	T4	KF318462.1
Ent-V09	Victoria	99	T4	N	T4-36	95	99	100	T4	KJ476515.1
Ent-V10	Victoria	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Ent-V11	Victoria		Contaminated							
Ent-V13	Victoria	100	T4	A	T4-36	98	100	100	T4	KF318462.1

*"Others" subtypes did not have close resemblance with any of the reference T4 subtypes.

Appendix-IC

Details of the soil samples processed on *Arcobacter* only for the isolation of *Acanthamoeba*

Soil Samples		Comparison with reference sequences					Comparison with Genbank sequences			
Code	Location	Max identity	T type	T4 subgroup	T4 subtype		Max identity	Coverage	T type claimed	Genbank Accession No.
					Nearest match	Identity (%)				
Arc-B01	Burnham	100	T4	E	T4-12	98	100	100	T4	JQ669660.2
Arc-B02	Burnham	99	T4	E	T4-12	98	100	99	T4	JQ669660.2
Arc-B03	Burnham	99	T4	E	T4-12	98	99	99	T4	KF928953.1
Arc-B04	Burnham	-	Not recovered	-	-	-	-	-	-	-
Arc-B05	Burnham	99	T4	N	Others*	90	99	100	T4	KF928946.1
Arc-B06	Burnham	99	T4	E	T4-12	100	100	100	T4	FJ422512.1
Arc-B07	Burnham	-	Not recovered	-	-	-	-	-	-	-
Arc-B08	Burnham	94	T2	-	-	-	98	100	T2	KF928936.1
Arc-B12	Burnham	99	T4	A	T4-35	98	99	100	T4	EU146070.1
Arc-B13	Burnham	100	T4	E	T4-12	98	100	99	T4	KF928953.1
Arc-E01	Edinburgh	99	T4	A	T4-31	98	100	99	T4	JX494783.1
Arc-E02	Edinburgh	-	Contaminated	-	-	-	-	-	-	-
Arc-E03	Edinburgh	98	T4	N	T4-36	95	99	99	T4	AM408796.1
Arc-E04	Edinburgh	99	T4	A	T4-31	98	100	100	T4	JX494772.1
Arc-E05	Edinburgh	99	T4	A	T4-22	100	99	100	T4	KJ094678.1
Arc-E06	Edinburgh	98	T4	N	Others*	88	99	100	T4	JQ678631.1
Arc-E07	Edinburgh	99	T4	E	T4-36	98	99	100	T4	GQ905497.1
Arc-E08	Edinburgh	98	T2	-	-	-	99	100	T2	AF019050.1
Arc-E09	Edinburgh	99	T4	B	T4-23	100	99	100	T4	KF928942.1
Arc-E10	Edinburgh	100	T2	-	-	-	100	100	T2	U07411.1
Arc-E11	Edinburgh	99	T4	E	T4-12	96	99	99	T4	HF930501.1
Arc-E12	Edinburgh	99	T4	B	T4-23	98	99	100	T4	KF928942.1
Arc-E13	Edinburgh	99	T4	B	T4-23	100	100	99	T4	JX423588.1

Arc-E14	Edinburgh	99	T4	N	Others*	88	99	99	T4	JX494771.1
Arc-E15	Edinburgh	100	T4	E	T4-12	98	100	100	T4	KF928953.1
Arc-E16	Edinburgh	100	T4	E	T4-12	98	99	100	T4	KF928953.1
Arc-E17	Edinburgh	100	T4	N	Others*	91/91	100	100	T4	KF928946.1
Arc-E18	Edinburgh	99	T4	E	T4-12	98	99	100	T4	JQ669660.2
Arc-E19	Edinburgh	100	T2	-	-	-	100	100	T2	U07411.1
Arc-E20	Edinburgh	100	T4	A	T4-31	100	100	99	T4	JX494783.1
Arc-E21	Edinburgh	99	T4	E	T4-12	96	100	100	T4	JQ669660.2
Arc-M01	Manchester	99	T4	A	T4-22	100	99	100	T4	KJ094678.1
Arc-M02	Manchester	99	T4	B	T4-23	100	99	100	T4	KF928942.1
Arc-M03	Manchester	99	T4	B	T4-23	100	100	99	T4	JX423588.1
Arc-M04	Manchester	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Arc-M05	Manchester	99	T4	E	T4-12	100	100	100	T4	FJ422512.1
Arc-M06	Manchester	100	T4	A	T4-31	100	100	99	T4	JX494783.1
Arc-M07	Manchester	100	T4	E	T4-12	98	99	100	T4	KF928953.1
Arc-M08	Manchester	-	Contaminated	-	-	-	-	-	-	-
Arc-M09	Manchester	-	Contaminated	-	-	-	-	-	-	-
Arc-M10	Manchester	-	Contaminated	-	-	-	-	-	-	-
Arc-NB01	North Berwick	99	T4	A	T4-31	98	100	99	T4	JX494783.1
Arc-NB02	North Berwick	99	T4	N	Others*	88	99	99	T4	JX494771.1
Arc-NB03	North Berwick	98	T2	-	-	-	99	100	T2	KF928936.1
Arc-NB04	North Berwick	98	T4	N	Others*	88	99	100	T4	JQ678631.1
Arc-NB05	North Berwick	99	T4	A	T4-31	98	100	100	T4	JX494772.1
Arc-NB06	North Berwick	98	T2/6	-	-	-	99	100	T2/6	AF019050.1
Arc-NB07	North Berwick	99	T4	E	T4-36	98	99	100	T4	GQ905497.1
Arc-NC01	Newcastle	98	T4	A	T4-35	98	99	100	T4	EU146070.1
Arc-NC02	Newcastle	98	T13/16	-	-	-	98	99	T16	AY026245.1
Arc-NC03	Newcastle	99	T4	N	Others*	91/91	100	99	T4	KJ094691.1
Arc-NC04	Newcastle	-	Not recovered	-	-	-	-	-	-	-
Arc-NC05	Newcastle	-	Not recovered	-	-	-	-	-	-	-
Arc-NC06	Newcastle	-	Not recovered	-	-	-	-	-	-	-

Arc-NC07	Newcastle	99	T4	B	T4-23	100	99	100	T4	KF928942.1
Arc-NC08	Newcastle	99	T4	B	T4-34	100	100	99	T4	DQ087324.1
Arc-NC09	Newcastle	98	T4	N	T4-36	95	99	99	T4	AM408796.1
Arc-P01	Paddington	-	Not recovered	-	-	-	-	-	-	-
Arc-P02	Paddington	99	T4	B	T4-23	100	100	99	T4	JX423588.1
Arc-P03	Paddington	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Arc-P04	Paddington	99	T2	-	-	-	99	100	T2	U07411.1
Arc-P05	Paddington	98	T4	B	T4-23	98	99	100	T4	KF928942.1
Arc-P06	Paddington	99	T4	A	T4-13	95	99	99	T4	JX494783.1
Arc-P07	Paddington	99	T4	B	T4-34	100	100	99	T4	KJ476527.1
Arc-P08	Paddington	-	Not recovered	-	-	-	-	-	-	-
Arc-P09	Paddington	97	T13	-	-	-	100	100	T13	KF928948.1
Arc-P11	Paddington	98	T2	-	-	-	99	99	T2	AF019050.1
Arc-S01	Slough	98	T13/16	-	-	-	98 (3/9)	99	T16	KF928941.1
Arc-S02	Slough	99	T4	E	T4-12	98	99	99	T4	KF928953.1
Arc-S03	Slough	-	Contaminated	-	-	-	-	-	-	-
Arc-S04	Slough	-	Not recovered	-	-	-	-	-	-	-
Arc-S05	Slough	-	Contaminated	-	-	-	-	-	-	-
Arc-S06	Slough	99	T4	A	T4-22	100	99	100	T4	KJ094678.1
Arc-S07	Slough	98	T2	-	-	-	99	100	T2	AF019050.1
Arc-SK01	S Kensington	99	T4	A	T4-35	98	99	100	T4	EU146070.1
Arc-SK02	S Kensington	100	T4	E	T4-12	98	99	100	T4	KF928953.1
Arc-SK03	S Kensington	99	T4	B	T4-34	100	100	99	T4	KJ476527.1
Arc-SK04	S Kensington	-	Not recovered	-	-	-	-	-	-	-
Arc-SK05	S Kensington	99	T4	E	T4-12	96	100	100	T4	JQ669660.2
Arc-SK06	S Kensington	98	T4	A	T4-35	98	99	100	T4	EU146070.1
Arc-SK07	S Kensington	98	T2/6	-	-	-	99	100	T2/T6	DQ087290.1
Arc-SK08	S Kensington	98	T4	B	T4-23	98	99	100	T4	KF928942.1
Arc-T01	Tilehurst	98	T2	-	-	-	99	100	T2	KF928936.1
Arc-T02	Tilehurst	99	T2	-	-	-	99	100	T2	U07411.1
Arc-T03	Tilehurst	99	T4	A	T4-13	95	99	99	T4	JX494783.1

Arc-T04	Tilehurst	100	T4	A	T4-36	98	100	100	T4	KF318462.1
Arc-T05	Tilehurst	99	T4	B	T4-34	100	100	99	T4	DQ087324.1
Arc-T06	Tilehurst	99	T4	E	T4-12	100	100	100	T4	FJ422512.1
Arc-T07	Tilehurst	-	Not recovered	-	-	-	-	-	-	-
Arc-T08	Tilehurst	98	T2	-	-	-	99	99	T2	AF019050.1
Arc-V01	Victoria	-	Not recovered	-	-	-	-	-	-	-
Arc-V02	Victoria	99	T4	N	Others*	92/92	100	99	T4	KJ094691.1
Arc-V03	Victoria	99	T4	E	T4-12	98	100	99	T4	JQ669660.2
Arc-V04	Victoria	100	T4	E	T4-12	98	100	99	T4	KF928953.1
Arc-V05	Victoria	-	Not recovered	-	-	-	-	-	-	-
Arc-V06	Victoria	100	T4	E	T4-12	98	100	100	T4	JQ669660.2
Arc-V07	Victoria	98	T4	E	T4-12	96	99	100	T4	DQ264391.1
Arc-V08	Victoria	100	T4	A	T4-31	100	100	99	T4	JX494783.1
Arc-V09	Victoria	98	T13	-	-	-	97(10/12)	100	T13	AF132134.1
Arc-V10	Victoria	99	T4	E	T4-12	96	99	99	T4	HF930501.1
Arc-V11	Victoria	100	T4	N	Others*	91/91	100	100	T4	KF928946.1
Arc-V13	Victoria	98	T2	-	-	-	99	100	T2	AF019050

*"Others" subtypes did not have close resemblance with any of the reference T4 subtypes.

Appendix-IIA

Thermotolerance and osmotolerance of the *Acanthamoeba* isolates on *E. coli* (Eco)

Sample		Thermotolerance			Osmo-tolerance (1M mannitol)
Code	**Location**	**23ºC**	**37ºC**	**41ºC**	
Eco-B01	Burnham	+++	+++	+	-
Eco-B02	Burnham	+++	+	-	-
Eco-B03	Burnham	+++	+++	+	-
Eco-B06	Burnham	+++	+++	+	-
Eco-B08	Burnham	+++	++	-	-
Eco-B12	Burnham	+++	++	-	-
Eco-B13	Burnham	+++	-	-	-
Eco-E01	Edinburgh	+++	+	-	-
Eco-E02	Edinburgh	+++	+	-	-
Eco-E03	Edinburgh	+++	+	-	-
Eco-E04	Edinburgh	+++	+++	+	-
Eco-E05	Edinburgh	+++	+	-	-
Eco-E06	Edinburgh	+++	++	-	-
Eco-E07	Edinburgh	+++	+	-	-
Eco-E08	Edinburgh	+++	+	-	-
Eco-E09	Edinburgh	+++	++	-	-
Eco-E10	Edinburgh	+++	+	-	-
Eco-E11	Edinburgh	+++	+	-	-
Eco-E12	Edinburgh	+++	++	-	-
Eco-E13	Edinburgh	+++	+	-	-
Eco-E14	Edinburgh	+++	+	-	-
Eco-E15	Edinburgh	+++	+	-	-
Eco-E16	Edinburgh	+++	+	-	-
Eco-E17	Edinburgh	+++	+	-	-
Eco-E18	Edinburgh	+++	+	-	-
Eco-E20	Edinburgh	+++	+	-	-
Eco-E21	Edinburgh	+++	+	-	-
Eco-M01	Manchester	+++	+	-	-
Eco-M02	Manchester	+++	++	-	-
Eco-M03	Manchester	+++	+	-	-
Eco-M04	Manchester	+++	+	-	-
Eco-M05	Manchester	+++	+	-	-
Eco-M06	Manchester	+++	+	-	-
Eco-M07	Manchester	+++	+	-	-
Eco-M08	Manchester	+++	+	-	-
Eco-NB01	N Berwick	+++	+	-	-
Eco-NB02	N Berwick	+++	-	-	-
Eco-NB03	N Berwick	+++	-	-	-
Eco-NB04	N Berwick	+++	++	-	-
Eco-NB05	N Berwick	+++	++	-	-
Eco-NB06	N Berwick	+++	+	-	-
Eco-NB07	N Berwick	+++	++	-	-
Eco-NC07	Newcastle	+++	+++	+	-
Eco-NC08	Newcastle	+++	++	-	-

Eco-NC09	Newcastle	+++	+	-	-
Eco-P02	Paddington	+++	+++	+	-
Eco-P03	Paddington	+++	+	-	-
Eco-P04	Paddington	+++	+	-	-
Eco-P05	Paddington	+++	+++	+	-
Eco-P06	Paddington	+++	+++	+	-
Eco-P07	Paddington	+++	++	-	-
Eco-P09	Paddington	+++	+	-	-
Eco-P11	Paddington	+++	++	-	-
Eco-S01	Slough	+++	++	-	-
Eco-S02	Slough	+++	+++	+	-
Eco-S03	Slough	+++	+	-	-
Eco-S05	Slough	+++	+	-	-
Eco-S06	Slough	+++	+	-	-
Eco-S07	Slough	+++	+	-	-
Eco-SK01	S Kensington	+++	+	-	-
Eco-SK02	S Kensington	+++	+	-	-
Eco-SK03	S Kensington	+++	++	-	-
Eco-SK05	S Kensington	+++	+	-	-
Eco-SK06	S Kensington	+++	++	-	-
Eco-SK07	S Kensington	+++	+	-	-
Eco-SK08	S Kensington	+++	+++	+	-
Eco-T01	Tilehurst	+++	-	-	-
Eco-T02	Tilehurst	+++	+	-	-
Eco-T03	Tilehurst	+++	+	-	-
Eco-T04	Tilehurst	+++	+	-	-
Eco-T05	Tilehurst	+++	+	-	-
Eco-T06	Tilehurst	+++	++	-	-
Eco-T08	Tilehurst	+++	+	-	-
Eco-V02	Victoria	+++	++	-	-
Eco-V03	Victoria	+++	+	-	-
Eco-V04	Victoria	+++	+	-	-
Eco-V06	Victoria	+++	+	-	-
Eco-V07	Victoria	+++	+	-	-
Eco-V08	Victoria	+++	-	-	-
Eco-V09	Victoria	+++	+	-	-
Eco-V10	Victoria	+++	+	-	-
Eco-V11	Victoria	+++	++	-	-
Eco-V13	Victoria	+++	++	-	-

Appendix-IIB

Thermotolerance and osmotolerance of the *Acanthamoeba* isolates on *Enterococcus* (Ent)

Sample		Thermotolerance			Osmo-tolerance (1M mannitol)
Code	**Location**	**23ºC**	**37ºC**	**40ºC**	
Ent-B01	Burnham	+++	+	-	-
Ent-B02	Burnham	+++	+++	+	-
Ent-B03	Burnham	+++	+	-	-
Ent-B06	Burnham	+++	+	-	-
Ent-B08	Burnham	+++	++	-	-
Ent-B12	Burnham	+++	++	-	-
Ent-B13	Burnham	+++	+	-	-
Ent-E01	Edinburgh	+++	+	-	-
Ent-E02	Edinburgh	+++	++	-	-
Ent-E03	Edinburgh	+++	+	-	-
Ent-E04	Edinburgh	+++	++	-	-
Ent-E05	Edinburgh	+++	+	-	-
Ent-E06	Edinburgh	+++	++	-	-
Ent-E07	Edinburgh	+++	+	-	-
Ent-E08	Edinburgh	+++	+	-	-
Ent-E09	Edinburgh	+++	++	-	-
Ent-E10	Edinburgh	+++	+	-	-
Ent-E11	Edinburgh	+++	+	-	-
Ent-E12	Edinburgh	+++	+++	+	-
Ent-E13	Edinburgh	+++	+	-	-
Ent-E14	Edinburgh	+++	+	-	-
Ent-E15	Edinburgh	+++	+	-	-
Ent-E16	Edinburgh	+++	+	-	-
Ent-E17	Edinburgh	+++	+	-	-
Ent-E18	Edinburgh	+++	+	-	-
Ent-E19	Edinburgh	+++	+	-	-
Ent-E20	Edinburgh	+++	+	-	-
Ent-E21	Edinburgh	+++	+	-	-
Ent-M01	Manchester	+++	+	-	-
Ent-M02	Manchester	+++	++	-	-
Ent-M03	Manchester	+++	++	-	-
Ent-M04	Manchester	+++	++	-	-
Ent-M05	Manchester	+++	+	-	-
Ent-M06	Manchester	+++	+	-	-
Ent-M07	Manchester	+++	+	-	-
Ent-M08	Manchester	+++	+	-	-
Ent-NB01	N Berwick	+++	+	-	-
Ent-NB02	N Berwick	+++	+	-	-
Ent-NB03	N Berwick	+++	+	-	-
Ent-NB04	N Berwick	+++	+	-	-
Ent-NB05	N Berwick	+++	++	-	-
Ent-NB06	N Berwick	+++	+	-	-
Ent-NB07	N Berwick	+++	++	-	-

Ent-NC01	Newcastle	+++	+	-	-
Ent-NC02	Newcastle	+++	+	-	-
Ent-NC03	Newcastle	+++	+	-	-
Ent-NC07	Newcastle	+++	++	-	-
Ent-NC08	Newcastle	+++	++	-	-
Ent-NC09	Newcastle	+++	+	-	-
Ent-P02	Paddington	+++	+	-	-
Ent-P03	Paddington	+++	+	-	-
Ent-P04	Paddington	+++	+	-	-
Ent-P05	Paddington	+++	++	-	-
Ent-P06	Paddington	+++	++	-	-
Ent-P07	Paddington	+++	++	-	-
Ent-P09	Paddington	+++	++	-	-
Ent-P11	Paddington	+++	-	-	-
Ent-S01	Slough	+++	-	-	-
Ent-S02	Slough	+++	+	-	-
Ent-S03	Slough	+++	+	-	-
Ent-S05	Slough	+++	+	-	-
Ent-S06	Slough	+++	+	-	-
Ent-S07	Slough	+++	+	-	-
Ent-SK01	S Kensington	+++	+	-	-
Ent-SK02	S Kensington	+++	+	-	-
Ent-SK05	S Kensington	+++	++	-	-
Ent-SK06	S Kensington	+++	++	-	-
Ent-SK07	S Kensington	+++	+	-	-
Ent-SK08	S Kensington	+++	-	-	-
Ent-T01	Tilehurst	+++	+	-	-
Ent-T02	Tilehurst	+++	++	-	-
Ent-T03	Tilehurst	+++	+	-	-
Ent-T04	Tilehurst	+++	+	-	-
Ent-T05	Tilehurst	+++	+	-	-
Ent-T06	Tilehurst	+++	+	-	-
Ent-T08	Tilehurst	+++	+	-	-
Ent-V02	Victoria	+++	+	-	-
Ent-V03	Victoria	+++	++	-	-
Ent-V04	Victoria	+++	+	-	-
Ent-V06	Victoria	+++	+	-	-
Ent-V07	Victoria	+++	+	-	-
Ent-V08	Victoria	+++	++	-	-
Ent-V09	Victoria	+++	+	-	-
Ent-V10	Victoria	+++	+	-	-
Ent-V13	Victoria	+++	+++	+	-

Appendix-IIC

Thermotolerance and osmotolerance of the *Acanthamoeba* isolates on *Arcobacter* (Arc)

Sample		Thermotolerance			Osmo-tolerance (1M mannitol)
Code	**Location**	**23ºC**	**37ºC**	**41ºC**	
Arc-B01	Burnham	+++	-	-	-
Arc-B02	Burnham	+++	+	-	-
Arc-B03	Burnham	+++	+	-	-
Arc-B05	Burnham	+++	+	-	-
Arc-B06	Burnham	+++	+	-	-
Arc-B08	Burnham	+++	+	-	-
Arc-B12	Burnham	+++	+	-	-
Arc-B13	Burnham	+++	+	-	-
Arc-E01	Edinburgh	+++	+	-	-
Arc-E03	Edinburgh	+++	+	-	-
Arc-E04	Edinburgh	+++	++	-	-
Arc-E05	Edinburgh	+++	+	-	-
Arc-E06	Edinburgh	+++	+	-	-
Arc-E07	Edinburgh	+++	++	+	-
Arc-E08	Edinburgh	+++	-	-	-
Arc-E09	Edinburgh	+++	+	-	-
Arc-E10	Edinburgh	+++	+	+	-
Arc-E11	Edinburgh	+++	-	-	-
Arc-E12	Edinburgh	+++	+	-	-
Arc-E13	Edinburgh	+++	+	-	-
Arc-E14	Edinburgh	+++	+	-	-
Arc-E15	Edinburgh	+++	+	-	-
Arc-E16	Edinburgh	+++	+	-	-
Arc-E17	Edinburgh	+++	+	-	-
Arc-E18	Edinburgh	+++	-	-	-
Arc-E19	Edinburgh	+++	+	+	-
Arc-E20	Edinburgh	+++	-	-	-
Arc-E21	Edinburgh	+++	+	-	-
Arc-M01	Manchester	+++	+	-	-
Arc-M02	Manchester	+++	++	-	-
Arc-M03	Manchester	+++	+	-	-
Arc-M04	Manchester	+++	+	-	-
Arc-M05	Manchester	+++	+	-	-
Arc-M06	Manchester	+++	+	-	-
Arc-M07	Manchester	+++	+	-	-
Arc-NB01	N Berwick	+++	+	-	-
Arc-NB02	N Berwick	+++	+	-	-
Arc-NB03	N Berwick	+++	+++	+	-
Arc-NB04	N Berwick	+++	+	-	-
Arc-NB05	N Berwick	+++	-	-	-
Arc-NB06	N Berwick	+++	-	-	-
Arc-NB07	N Berwick	+++	++	+	-
Arc-NC01	Newcastle	+++	+	-	-
Arc-NC02	Newcastle	+++	+	-	-

Arc-NC03	Newcastle	+++	+	-	-
Arc-NC07	Newcastle	+++	++	-	-
Arc-NC08	Newcastle	+++	+	-	-
Arc-NC09	Newcastle	+++	+	-	-
Arc-P02	Paddington	+++	+	-	-
Arc-P03	Paddington	+++	+	-	-
Arc-P04	Paddington	+++	+	-	-
Arc-P05	Paddington	+++	+	-	-
Arc-P06	Paddington	+++	++	-	-
Arc-P07	Paddington	+++	+	-	-
Arc-P09	Paddington	+++	+	-	-
Arc-P11	Paddington	+++	-	-	-
Arc-S01	Slough	+++	-	-	-
Arc-S02	Slough	+++	+	-	-
Arc-S06	Slough	+++	+	-	-
Arc-S07	Slough	+++	+	-	-
Arc-SK01	S Kensington	+++	+	-	-
Arc-SK02	S Kensington	+++	+	-	-
Arc-SK03	S Kensington	+++	+	-	-
Arc-SK05	S Kensington	+++	+	-	-
Arc-SK06	S Kensington	+++	+	-	-
Arc-SK07	S Kensington	+++	-	-	-
Arc-SK08	S Kensington	+++	-	-	-
Arc-T01	Tilehurst	+++	+++	+	-
Arc-T02	Tilehurst	+++	-	-	-
Arc-T03	Tilehurst	+++	+	-	-
Arc-T04	Tilehurst	+++	+	-	-
Arc-T05	Tilehurst	+++	+	-	-
Arc-T06	Tilehurst	+++	+	-	-
Arc-T08	Tilehurst	+++	-	-	-
Arc-V02	Victoria	+++	+	-	-
Arc-V03	Victoria	+++	+	-	-
Arc-V04	Victoria	+++	+	-	-
Arc-V06	Victoria	+++	-	-	-
Arc-V07	Victoria	+++	+	-	-
Arc-V08	Victoria	+++	+	-	-
Arc-V09	Victoria	+++	-	-	-
Arc-V10	Victoria	+++	-	-	-
Arc-V11	Victoria	+++	+	-	-
Arc-V13	Victoria	+++	+	-	-

Appendix-IIIA

Details of the bacterial endosymbionts detected in the *Acanthamoeba* isolates of Eco

Sample		Acanthamoeba genotype			Bacterial endosymbionts genotype			
Code	**Location**	**T type**	**T4 subgroup**	**T4 subtype**	**Max identity**	**Coverage**	**Bacterial endosymbionts**	**Genbank Accession No.**
Eco-E06	Edinburgh	T4	N	T4-36	99	100	*Candidatus* Procabacter	KF924592.1
Eco-E09	Tilehurst	T4	B	T4-23	99	100	*Candidatus* Procabacter	KF924592.1
Eco-E13	Tilehurst	T4	B	T4-23	99	100	*Candidatus* Procabacter	KF924592.1
Eco-E21	Tilehurst	T4	B	T4-23	99	100	*Candidatus* Procabacter	KF924592.1
Eco-SK03	Edinburgh	T4	N	T4-36	99	100	*Candidatus* Procabacter	KF924592.1
Eco-T05	Tilehurst	T4	B	T4-23	99	100	*Candidatus* Procabacter	KF924592.1

Appendix-IIIB

Details of the bacterial endosymbionts detected in the *Acanthamoeba* isolates of Ent

Sample		Acanthamoeba genotype			Bacterial endosymbionts genotype			
Code	**Location**	**T type**	**T4 subgroup**	**T4 subtype**	**Max identity**	**Coverage**	**Bacterial endosymbionts**	**Genbank Accession No.**
Ent-M02	Manchester	T4	B	T4-23	99	99	*Candidatus* Procabacter sp.	AF177426.1
Ent-NB01	North Berwick	T4	B	T4-23	99	99	*Candidatus* Procabacter sp.	AF177426.1
Ent-NB07	North Berwick	T4	B	T4-23	99	99	*Candidatus* Procabacter sp.	AF177426.1
Ent-NC01	Newcastle	T4	A	T4-22	99	100	Uncultured bacterium clone CN1-93	HQ218746.1
Ent-NC07	Newcastle	T4	B	T4-23	99	99	*Candidatus* Procabacter sp.	AF177426.1
Ent-P02	Paddington	T4	A	T4-22	99	100	Uncultured bacterium clone CN1-93	HQ218746.1
Ent-P04	Paddington	T4	E	T4-12	99	99	*Candidatus* Procabacter sp.	AF177425.1
Ent-P06	Paddington	T4	A	T4-36	99	99	*Candidatus* Procabacter	KF924592.1
Ent-S06	Slough	T4	A	T4-22	99	100	Uncultured bacterium clone CN1-93	HQ218746.1
Ent-T05	Tilehurst	T4	B	T4-23	99	99	*Candidatus* Procabacter sp.	AF177426.1
Ent-V06	Victoria	T4	B	T4-23	99	99	*Candidatus* Procabacter sp.	AF177426.1

Appendix-IIIC

Details of the bacterial endosymbionts detected in the *Acanthamoeba* isolates of Arc

Sample		Acanthamoeba genotype			Bacterial endosymbionts genotype			
Code	**Location**	**T type**	**T4 subgroup**	**T4 subtype**	**Max identity**	**Coverage**	**Bacterial endosymbionts**	**Genbank Accession No.**
Arc-B05	Burnham	99	T4	N	Others*	93	*Massilia* sp. Br15	KC442331.1
Arc-B06	Burnham	99	T4	E	T4-12	99	*Candidatus* Procabacter sp. P23	AF177425.1
Arc-B12	Burnham	99	T4	A	T4-35	99	*Candidatus* Procabacter sp. UWC6	AF177426.1
Arc-E06	Edinburgh	98	T4	N	Others*	99	*Candidatus* Procabacter sp. UWC6	AF177426.1
Arc-E12	Edinburgh	99	T4	B	T4-23	99	*Candidatus* Procabacter sp. UWC6	AF177426.1
Arc-M05	Manchester	99	T4	E	T4-12	99	*Candidatus* Procabacter sp. P23	AF177425.1
Arc-NB04	North Berwick	98	T4	N	Others*	99	*Candidatus* Procabacter sp. UWC6	AF177426.1
Arc-P05	Paddington	98	T4	B	T4-23	99	*Candidatus* Procabacter sp. UWC6	AF177426.1
Arc-P11	Paddington	98	T2	-	-	100	Uncultured bacterium clone N-08	HQ218452.1
Arc-SK01	S Kensington	99	T4	A	T4-35	99	*Candidatus* Procabacter sp. UWC6	AF177426.1
Arc-SK05	S Kensington	99	T4	E	T4-12	99	*Candidatus* Procabacter sp. UWC6	AF177426.1
Arc-SK08	S Kensington	98	T4	B	T4-23	99	*Candidatus* Procabacter sp. UWC6	AF177426.1
Arc-T08	Tilehurst	98	T2	-	-	100	Uncultured bacterium clone N-08	HQ218452.1

*"Others" subtypes did not have close resemblance with any of the reference T4 subtypes.